Changes in High Density Lipoprotein Subfraction Lipids During Neutral Lipid Transfer in Healthy Subjects and in Patients with Insulin-Dependent Diabetes Mellitus

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ABSTRACT: While it is known that the transfer of cholesteryl ester (CE) from high density lipoprotein (HDL) to the apo B-containing lipoproteins is increased in patients with diabetes, the extent to which the various lipoprotein fractions engage in neutral lipid exchange and the magnitude to which triglyceride (TG) is translocated is not known. To examine in greater detail neutral lipid net mass transfer in diabetes, the HDL subfractions and the apo B-containing lipoproteins were separated, and the net mass transfer of CE and TG was compared to that of control subjects. In both groups, bidirectional transfer of CE from HDL_3 to very low density lipoprotein (VLDL) + low density lipoprotein (LDL) and of TG from VLDL + LDL to HDL_3, took place, but this process was significantly greater ($P < .01$) in insulin-dependent diabetes mellitus (IDDM). In contrast, CE and TG accumulated in HDL_2 to a similar degree in normal and IDDM subjects. In recombination experiments with each of the apo B-containing lipoproteins, IDDM VLDL had a greater capacity to facilitate the exchange of core lipids from both IDDM and control HDL_3: on the other hand, LDL from IDDM and control subjects both donated TG and CE to HDL_2 and affected little change in HDL_3. These findings indicate that all the major plasma fractions normally participate in the trafficking of CE and TG among the lipoproteins during neutral lipid transfer and show that the principal perturbation in cholesteryl ester transfer in IDDM involves altered interaction between VLDL and the HDL_3 subfraction. *Lipids 31*, 1–7 (1996).

Specialized proteins in human plasma catalyze the transfer and exchange of cholesteryl ester (CE), triglyceride (TG), and phospholipids between lipoproteins (1–3). The reactions mediated by these lipid transfer proteins represent an important pathway in the intravascular metabolism of lipoproteins. Consequently, disturbances in transfer may not only adversely affect the composition of lipoproteins, but under certain circumstances they may also alter their metabolism. Plasma CE transfer protein (CETP) displays CE, TG, retinyl ester, and phospholipid transfer activities (2,4). Since it mediates all of the neutral lipid transfer activity in plasma, CETP plays a prominent role in the centripetal transport of CE from high density lipoprotein (HDL) to other lipoproteins and its reutilization by other tissues, and in its ultimate removal and excretion by the liver.

We have recently reported that both the net mass and isotopic transfer of CE from HDL to the apo B-containing lipoproteins is increased in plasma from normolipidemic insulin-dependent diabetes mellitus (IDDM) patients receiving conventional subcutaneous insulin treatment (5,6). While these studies implied that accelerated CET may be a previously unrecognized abnormality that increases cardiovascular risk in diabetes, they did not reveal the extent to which the HDL subfractions engaged in the transfer reaction and whether TG transfer also was increased. To obtain more detailed information about the roles played by the individual HDL subfractions and the behavior of very low density lipoprotein (VLDL) and low density (LDL) during CET in IDDM, we have studied the redistribution of HDL_2 and HDL_3 core lipids in intact plasma and in the presence of VLDL and LDL alone from control and IDDM subjects.

MATERIALS AND METHODS

Human subjects. Five IDDM patients (four female, one male) and five normolipidemic controls (three female, two male) were studied. The study protocol was approved by the Institutional Human Investigation Committee. Informed consent was obtained and the project was carried out according to the principles of the Declaration of Helsinki. Classification of these patients as having IDDM was based on clinical criteria only. Most had a prior history of ketoacidosis. All required insulin therapy to prevent ketosis. All patients followed a standard American Diabetes Association diet before study. None had renal disease manifested as proteinuria (Clinistix) or an elevated serum creatinine level, infection, was a vegetarian, cigarette smoker, an athlete, took drugs known to affect lipid metabolism, or had a history of ketoacidosis within six months prior to study. Control subjects were healthy, took no medications, and were matched for sex, approximate age, fasting lipid levels, weight, and level of physical activity. Blood for all studies was collected after an overnight fast in Na-EDTA-containing tubes, and plasma was separated promptly by low-speed centrifugation.

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Abbreviations: CE, cholesteryl ester; CET, cholesteryl ester transfer; CETP, cholesteryl ester transfer protein; HDL, high density lipoprotein; IDDM, insulin-dependent diabetes mellitus; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; TG, triglyceride; VLDL, very low density lipoprotein.

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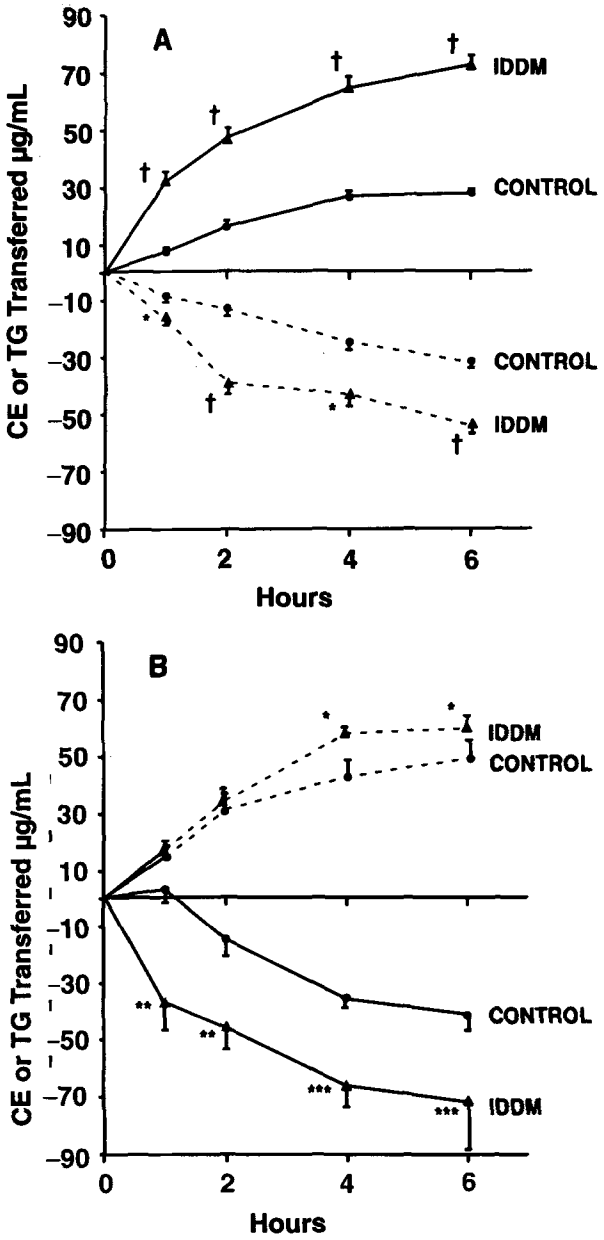


FIG. 1. Changes in the mass in cholesteryl ester (CE) and triglyceride (TG) of very low density lipoprotein (VLDL) + low density lipoprotein (A) and high density lipoprotein (HDL) (B) during transfer in intact plasma in five insulin-dependent diabetes mellitus (IDDM) and five control subjects; — CE; – – TG; *$P < .05$; **$P < .025$; ***$P < .01$; †$P < .001$.

CET. The mass transfer of CE, which measures the net transfer of preformed CE from HDL to VLDL + LDL in 1 mL of intact plasma, was measured during incubation at 37°C in a metabolic shaker in the presence 1.5 mM dithio-*bis*-5 dinitrobenzoic acid to inhibit lecithin cholesterol acyltransferase as previously described (7). Briefly, aliquots of plasma were removed prior to and after 1, 2, 4, and 6 h of incubation, chilled on ice, and then HDL_2 and HDL_3 were separated from VLDL and/or LDL by differential precipitation with dextran sulfate (8). At each sampling interval, the amounts of free and total cholesterol present in the supernatant were measured, and the amount of CE transferred into the apo B-containing lipoproteins was calculated from the difference between the two values.

Lipoprotein isolation. For the recombination studies, VLDL (d < 1.006 g/mL) and intermediate density lipoprotein (IDL)–LDL (d 1.006–1.063 g/mL) were isolated by sequential preparative ultracentrifugation at 8°C in a 40.3 rotor at 40,000 rpm for 18 h.

Analytical methods. Enzymatic kit procedures were employed to quantitate glucose (Boehringer-Mannheim, Indianapolis, IN), fructosamine (9), cholesterol (Boehringer-Mannheim), and TG (Sigma Chemical, St. Louis, MO). HDL–cholesterol was measured by a Standardized Lipid Research Clinic heparin/$MnCl_2$ precipitation procedure (10). Free cholesterol was estimated with the same components of the cholesterol kit except that CE hydrolase was omitted. CE was determined from the difference between total and free cholesterol.

Statistical methods. The means for each variable studied in diabetic and control subjects were compared by Student's *t*-test.

RESULTS

While fasting plasma lipids were similar in the two groups (Table 1), glucose and fructosamine were both significantly increased in the IDDM subjects. During the incubation of plasma, the extent to which IDDM VLDL + LDL acquired CE from HDL and donated TG to HDL (Fig. 1) was significantly increased. In both diabetic and control groups, the major interaction in plasma occurred between the apo B-containing lipoproteins and the HDL_3 subfraction (Fig. 2). Here too the magnitude of the bidirectional movement of core lipids differed in IDDM and control subjects. No striking sex difference was observed in the CET response within each small group. While CE declined with time in HDL_3 in both groups, it fell in IDDM HDL_3 more rapidly and to a significantly greater extent than in control HDL_3. At the same time, TG accrued at an increased rate and to a greater degree in IDDM HDL_3 than in controls. These changes together indicate that there was a greater net increment in TG and loss of CE in IDDM than in control HDL_3. In contrast, CE and TG both increased to a similar degree in their HDL_2 (Fig. 3).

Role of VLDL. To distinguish the contributions made by VLDL to these changes in the HDL subfractions, we performed a series of recombination experiments. Combining control VLDL with diabetic d > 1.063 g/mL fractions altered

TABLE 1
Clinical Features[a]

	Control	IDDM
Fasting glucose (mg/dl)	83.2 ± 3.4	192.3 ± 90.7[b]
Fructosamine (umoles/L)	209 ± 31.6	366 ± 34.9[c]
Triglycerides (mg/dl)	96.8 ± 29.5	112.5 ± 19.9
Cholesterol (mg/dl)	170.4 ± 26.3	179.0 ± 87.8
HDL (mg/dl)	47.4 ± 4.9	48.5 ± 8.5

[a](mean ± SD); n = 5; [b]$P < .05$; [c]$P < .001$; IDDM, insulin-dependent diabetes mellitus; HDL, high density lipoprotein.

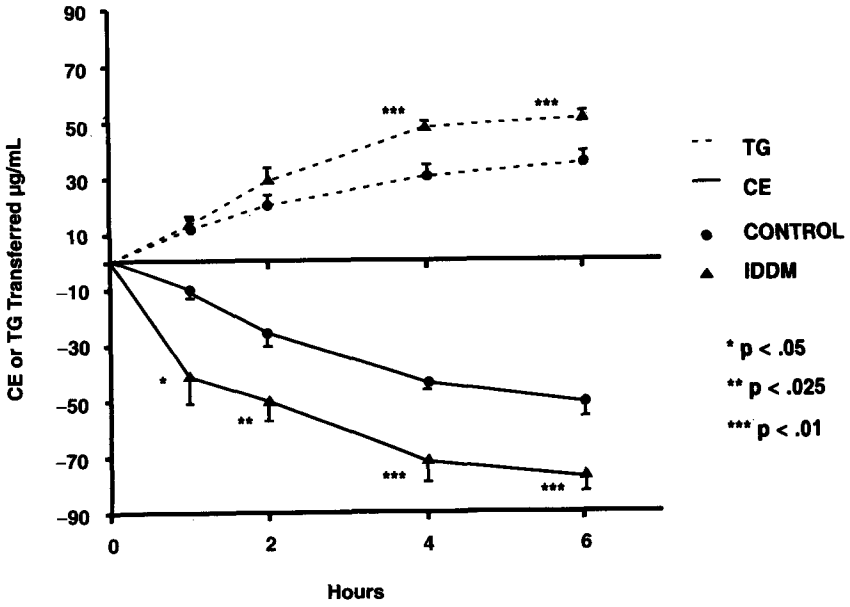


FIG. 2. Changes in the mass of CE and TG in HDL_3 during CE transfer in intact plasma in five IDDM (▲) and five control (●) subjects. See Figure 1 for abbreviations and other symbols.

the VLDL core lipids in a manner identical to that observed when VLDL from two controls were added at the same TG concentration to their own d > 1.063 g/mL fractions (Fig. 4A). These data indicate that this IDDM HDL fraction had a normal capacity to stimulate neutral lipid transfer. In contrast, the change in IDDM VLDL core lipid content was virtually identical when it was combined with IDDM and control d > 1.063 g/mL fractions (Fig. 4B). This finding demonstrates that IDDM VLDL had an enhanced capacity to stimulate CET. In these experiments, TG and CE concentrations were similar in HDL_2 in the two groups (Table 2), suggesting that the abnormal interaction between IDDM VLDL and the d < 1.063 g/mL fraction involved only HDL_3. Indeed, a profile of net mass transfer was found in HDL_3 (Fig. 5) which was the reciprocal of that observed for VLDL (Fig. 4). This finding implies that the changes observed in CET in whole plasma largely reflected the interaction between VLDL and HDL_3.

Role of IDL–LDL. When added at the same concentration of cholesterol, both IDDM and control IDL–LDL donated CE and TG to HDL_2 though this property was somewhat greater in control than in IDDM IDL–LDL (Fig. 6). In contrast, little net mass transfer of either CE or TG was observed in HDL_3 when IDL–LDL from IDDM and controls were combined with d < 1.063 g/mL fractions (Table 3).

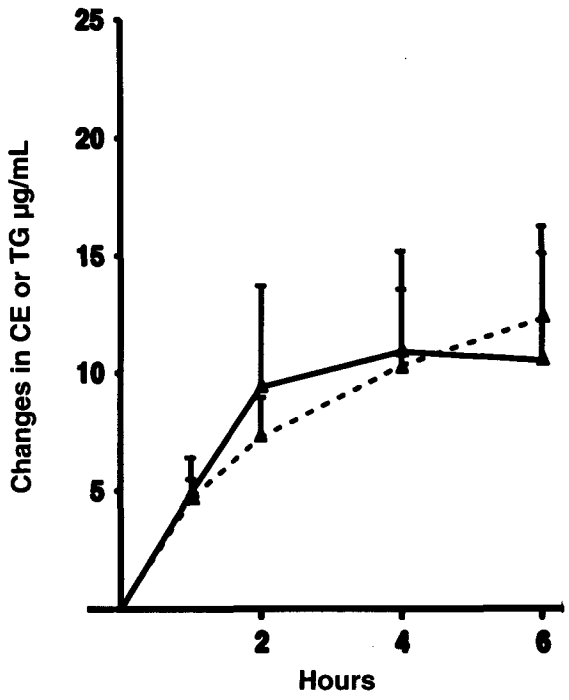
DISCUSSION

The HDL subfractions play a number of key roles in the centripetal movement of cholesterol from peripheral tissues to the liver (11). The smallest HDL, the prebeta-migrating fraction present in the HDL_3 density range, not only promote the efflux of cellular cholesterol, but they appear to be the major site for its esterification by lecithin cholesterol acyltransferase (12). In addition, data in the present study obtained in intact human plasma corroborate recent work performed in isolated and partially reconstituted experimental systems, showing that particles in the HDL_3 density range also are the main

TABLE 2
Changes in Core Lipid Content of HDL_2[a]

	CE transferred to HDL_2			
	1	2	4	6
	Hours			
$VLDL_c$ + d 1.063g/mL Bottom$_c$	2.3 ± 4.0	2.3 ± 0.9	−0.45 ± 1.1	−1.1 ± 4.1
$VLDL_c$ + d 1.063g/mL Bottom$_{IDDM}$	−0.7 ± 1.1	−0.2 ± 0.2	−0.6 ± 1.1	−2.0 ± 3.2
$VLDL_{IDDM}$ + d 1.063 g/mL Bottom$_c$	−1.7 ± 0.14	−3.0 ± 1.8	−1.5 ± 0.26	−5.0 ± 3.2
$VLDL_{IDDM}$ + d 1.063 g/mL Bottom$_{IDDM}$	−2.1 ± 4.7	−2.4 ± 4.1	4.3 ± 1.9	−2.2 ± 0.14
	TG transferred to HDL_2			
	1	2	4	6
	Hours			
$VLDL_c$ + d 1.063 g/mL Bottom$_c$	3.2 ± 3.2	3.1 ± 4.3	2.8 ± 0.6	3.2 ± 3.2
$VLDL_c$ + d 1.063 g/mL Bottom IDDM	5.8 ± 1.2	0 ± 1.0	-4.6 ± 4.2	2.3 ± 3.5
$VLDL_{IDDM}$ + d 1.063 g/mL Bottom$_c$	2.0 ± 1.98	3.0 ± 0.80	2.0 ± 1.98	1.4 ± 2.6
$VLDL_{IDDM}$ + d 1.063 g/mL Bottom$_{IDDM}$	2.8 ± 2.8	4.4 ± 2.1	0.55 ± 1.98	3.25 ± 2.3

[a]Conditions: Very low density lipoprotein (VLDL) from four IDDM and four control subjects were combined at plasma concentration with d 1.063 g/mL bottom fractions (ug/mL plasma); CE, cholesteryl ester; TG, triglyceride; see Table 1 for other abbreviations.



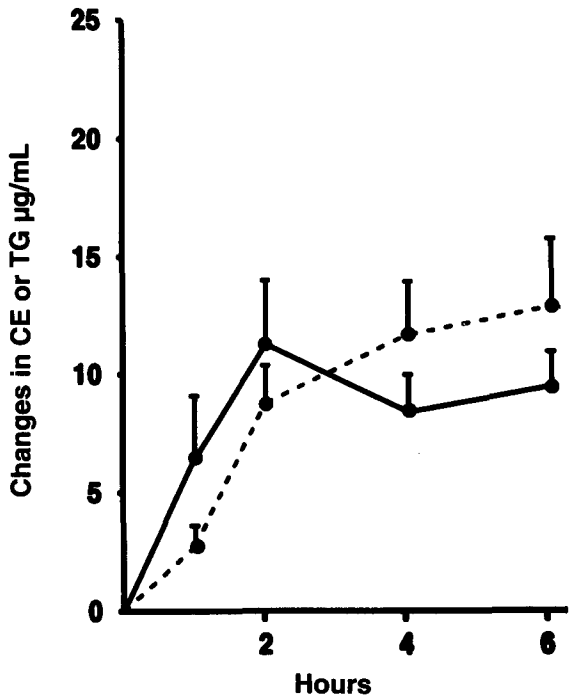
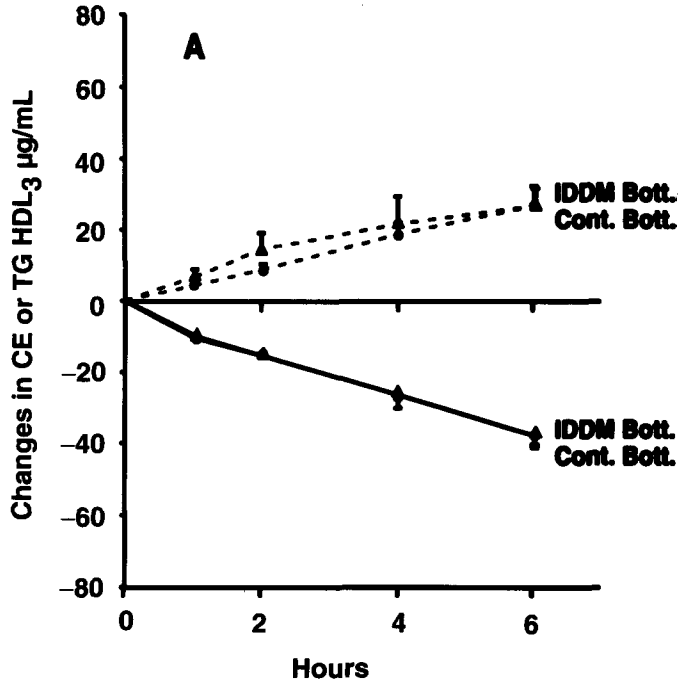


FIG. 3. Changes in the mass of CE and TG in HDL_2 during CE transfer in intact plasma in (A) five IDDM and (B) five control subjects. See Figures 1 and 2 for symbols and abbreviations.



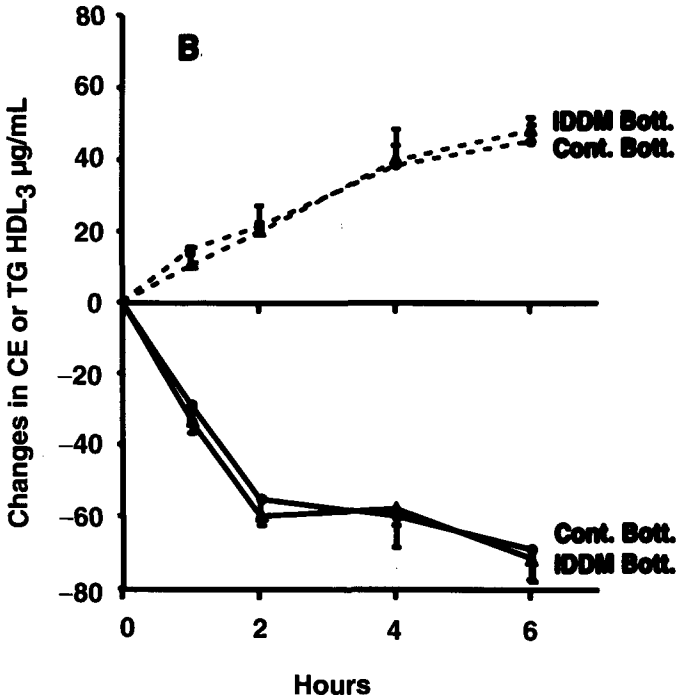


FIG. 4. Changes in the mass of CE and TG in VLDL when VLDL (d < 1.006 g/mL fraction) from two control (Cont.) (A) and two IDDM (B) subjects were combined at plasma concentration with d > 1.063 g/mL plasma fractions as indicated; Bott., bottom. See Figures 1 and 2 for abbreviations and symbols.

reservoir in plasma from which esterified cholesterol (CE) is transferred to the apo B-containing lipoproteins (13). This central step in reverse cholesterol transport, designated CET (2–4), is mediated by the 74 kd protein CETP that has been shown to be closely associated with smaller HDL particles (14).

A number of different lines of evidence in humans and experimental animals suggest that, when CETP activity is increased, the atherogenicity of VLDL and LDL is enhanced because they become enriched with CE (3). The lipoprotein profile in pedigrees of families with CETP deficiency (in-

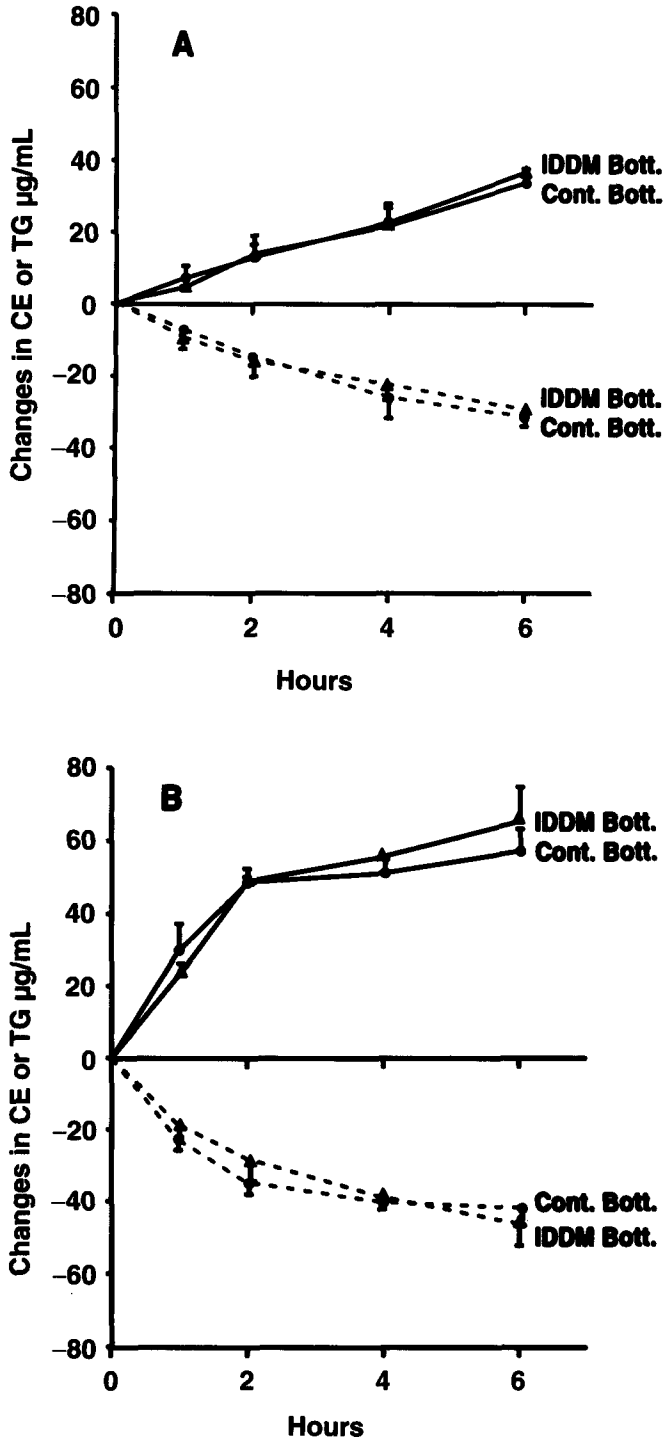


FIG. 5. Changes in the mass of CE and TG in HDL_3 when VLDL (d < 1.006 g/mL) from two control (A) and two IDDM (B) subjects were combined with d > 1.063 g/mL plasma fractions as indicated. See Figures 1, 2, and 4 for abbreviations and symbols.

creased HDL, a decrease in the CE content of the apo B-containing lipoproteins—VLDL, IDL, LDL, and decreased apo B) is typically one associated with resistance to atherosclerosis and some CETP-deficient families appear to experience longevity (15). Moreover, those animal species with low levels of CETP activity in plasma such as the rat, cow, and dog are resistant to dietary-induced atherosclerosis; those that have active CET such as rabbits, monkeys, and humans are highly susceptible (16). When mice, a resistant species, are made transgenic for simian CETP, they develop atherosclerosis in response to dietary fat (17). In contrast, those conditions in humans in which the rate of net transfer of CE is increased such as dysbetalipoproteinemia (18), and both IDDM (5,19) and noninsulin-dependent diabetes mellitus (20), all are associated with an acceleration of atherogenesis.

TABLE 3
Changes in Core Lipid Content of HDL_3[a]

	CE transferred to HDL_2			
	1	2	4	6
	Hours			
LDL_c + d 1.063g/mL Bottom$_c$	2.7 ± 5.2	6.9 ± 0.9	2.3 ± 0.3	3.2 ± 2.6
LDL_c + d 1.063g/mL Bottom$_{IDDM}$	2.4 ± 1.7	5.5 ± 1.3	1.2 ± 0.1	−6.5 ± 1.8
LDL_{IDDM} + d 1.063 g/mL Bottom$_c$	1.3 ± 2.7	−3.7 ± 3.3	−5.7 ± 5.3	0.3 ± 0.3
LDL_{IDDM} + d 1.063 g/mL Bottom$_{IDDM}$	−2.5 ± 2.5	−4.5 ± 1.99	−2.2 ± 1.9	−1.6 ± 7.7
	TG transferred to HDL_2			
	1	2	4	6
	Hours			
LDL_c + d 1.063 g/mL Bottom$_c$	4.0 ± 4.0	1.2 ± 1.1	0	−0.5 ± 0.5
LDL_c + d 1.063 g/mL Bottom$_{IDDM}$	−1.5 ± 1.5	−1.0 ± 0.9	−2 ± 0	−3.4 ± 1.3
LDL_{IDDM} + d 1.063 g/mL Bottom$_c$	−1.9 ± 3.9	−2.8 ± 1.8	−4.6 ± 4.6	−2.25 ± 2.3
LDL_{IDDM} + d 1.063 g/mL Bottom$_{IDDM}$	2.25 ± 2.3	1.65 ± 1.6	0	−0.5 ± 0.6

[a]Conditions: low density lipoprotein (LDL) from four IDDM and four control subjects were combined at plasma concentration with d 1.063 g/mL bottom fractions (ug/mL plasma). See Tables 1 and 2 for other abbreviations.

We have shown earlier that CE was transferred at an increased rate from HDL to VLDL and LDL in IDDM and noninsulin-dependent diabetes mellitus, and now again observe that altered behavior of VLDL was responsible for this disturbance. In our earlier studies we reported only the changes in CE and not in TG in the entire HDL fraction. Consequently, it was unclear to what extent HDL_2 and HDL_3 engaged in CET and the degree to which their respective core lipids were altered in the process. In the present study, we show for the first time that under the conditions of our assay there is a net increase in core lipids in HDL_2 and a net loss in HDL_3 during CET.

We confirm data obtained with reconstituted systems and demonstrate that the HDL_3 subfraction interacts predominantly with CETP and is not only the principal donor of CE to the apo B-containing lipoproteins, but also the primary acceptor of TG from VLDL. Moreover, our recombination studies demonstrate that the differences in CET in IDDM and control plasma are largely attributable to enhanced trafficking of

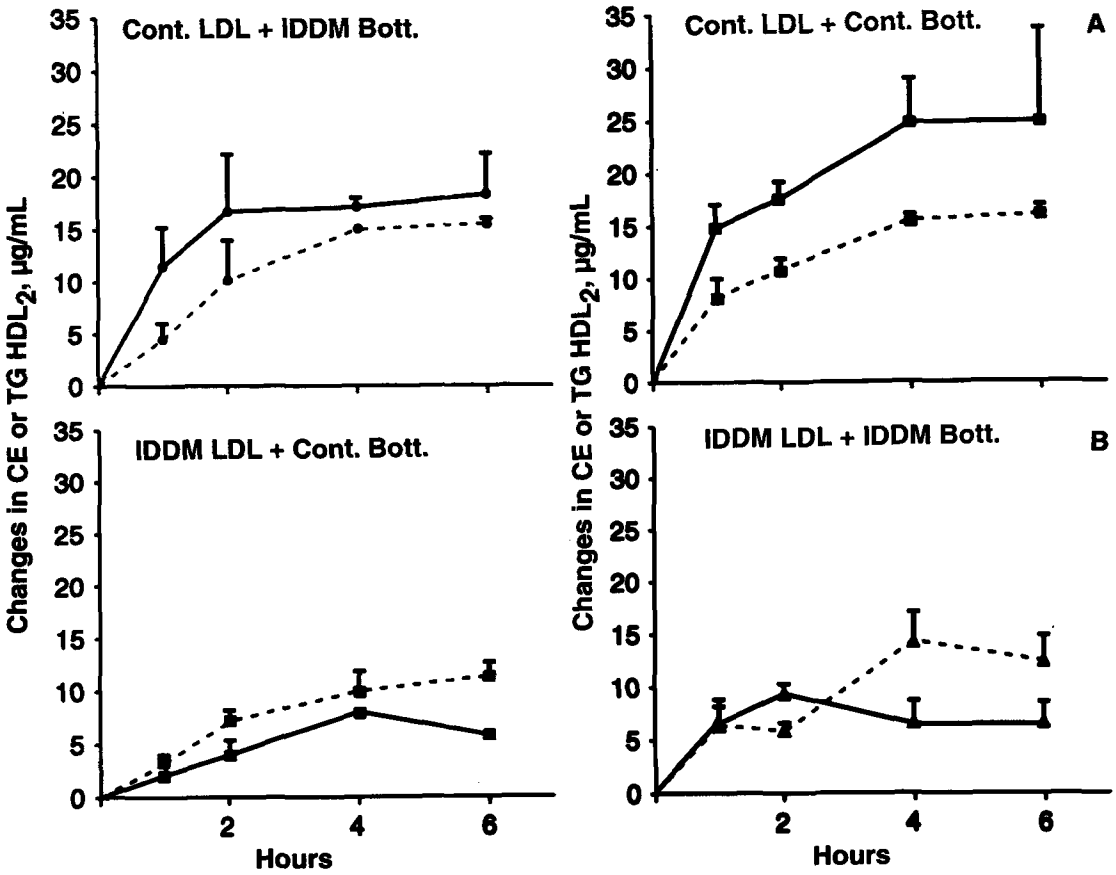


FIG. 6. Changes in the mass of HDL_2 core lipids when intermediate density lipoprotein (IDL)–LDL (d 1.006–1.063 g/mL) from two control (A) and two IDDM (B) subjects were combined with d > 1.063 g/mL plasma fractions as indicated. See Figures 1, 2, and 4 for abbreviations and symbols.

core lipids between VLDL and the HDL_3 subfraction. Since these experiments with VLDL were performed at the same TG concentration and the IDDM patients and controls were normolipidemic, it is unlikely that differences in particle number were responsible for the changes we found. Having not measured VLDL apo B, however, it is still possible that a small increase in VLDL particle number contributed to the increase in CET observed with this isolated fraction under these conditions. It seems more likely, however, that this behavior results from the presence of a subpopulation of lipoprotein particles in the IDDM VLDL fraction with distinctive alterations in lipoprotein composition likely accounting for this functional change. Although Morton (21) has shown that enrichment of lipoproteins with free cholesterol enhances their interaction with CETP and we have reported that this alteration manifested by an increase in the free cholesterol/lecithin ratio is present in diabetic VLDL and LDL (22), the precise cause of this altered behavior of VLDL during CET is still unclear.

Barter and co-workers (22) have shown in incubation studies performed with isolated HDL_3 that exposure to CETP resulted in the formation of populations of both larger and smaller particles. If an effect of this type occurred in the present studies, it could confound our results by altering the distribution of HDL particles in the HDL_2 and HDL_3 subfractions. This seems unlikely to have occurred, however, as later experiments from the same group showed, when apo B-containing particles were present (VLDL, LDL), the larger particles found in an isolated system did not form (22); instead, increased numbers of smaller particles were observed. If this latter effect of CETP occurred in incubated intact plasma in the present study, it would not affect our results because particles in HDL_3 that engaged in CET would remain in this fraction.

In contrast to the differences shown in the level of interaction of IDDM and control HDL_3 and VLDL, their HDL_2 subfractions functioned to the same degree as avid acceptors of both TG and CE. The propensity of the entire HDL fraction to accrue CE during the initial phase of CET in normal subjects was described earlier by VanTol and co-workers (23). Here we extend their observations: first, we show that the increase in CE mass involves the HDL_2 subfraction only; secondly, we find that the increment in CE is accompanied by a simultaneous parallel increase in TG.

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Hydrocarbon Chain Distribution of Ether Phospholipids of the Ascidian *Halocynthia roretzi* and the Sea Urchin *Strongylocentrotus intermedius*

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ABSTRACT: The contents and compositions of the 1-*O*-alk-1'-enyl-2-acyl, 1-*O*-alkyl-2-acyl, and 1,2-diacyl glycerophospholipids in the muscle and viscera of the ascidian *Halocynthia roretzi*, and of the gonad of the sea urchin *Strongylocentrotus intermedius*, which are eaten to some extent in Alaska and in Asia, were analyzed by gas–liquid chromatography. 1-*O*-Alk-1'-enyl-2-acyl glycerophospholipids were found in all of the samples, accounting for 64.4–69.0% of the ethanolamine glycerophospholipid (EPL). By contrast, the levels of the 1-*O*-Alk-1'-enyl-2-acyl choline glycerophospholipids (CPL) were low (3.1–5.7%). CPL was rich in the 1-*O*-alkyl-2-acyl subclass amounting to 12.5–23.9% in the ascidian sample. The level of CPL in the sea urchin gonad was extremely high, amounting to 46.1%. The most prominent alkyl chains in the *sn*-1 position of CPL from the ascidian muscle were 16:0 (44.6%), 18:1 (26.5%), and 18:0 (10.7%), and of CPL from the sea urchin gonad were 18:0 (36.2%), 16:0 (33.0%), and 18:1 (17.8%). Unusually high levels of odd-numbered alkyl chains, e.g., 19:0 and *anteiso* 17:0, were detected in the CPL of all samples. The prominent alkenyl chains of EPL were 18:0 (69.4%), 16:0 (10.0%), and 18:1 (8.54%) (not counting the vinyl double bond) for the sea urchin gonad. Relatively high levels of 20:1 alkenyl chains were also present. The glycerol *sn*-2 positions contained high proportions of polyunsaturated fatty acids. Thus, 20:5n-3 (43.6%) and 22:6n-3 (20.1%) were most abundant in the alkylacyl CPL from the ascidian muscle and 20:5n-3 (54.9%) and 20:4n-6 (30.1%) in alkylacyl CPL from the sea urchin gonad. Despite a possible interconversion of the alkyl and alkenyl chains of each class of the ether phospholipids, they showed few features in common. *Lipids 31*, 9–18 (1996).

Marine invertebrates are rich sources of ether lipids, i.e., alkenylacyl glycerophosphocholines and glycerophosphoethanolamines (plasmalogens) and alkylacyl glycerophosphocholines and glycerophosphoethanolamines. Alkenylacyl choline glycerophospholipids (CPL) and ethanolamine glycerophospholipids (EPL) in marine invertebrates tissues are accompanied by a very significant amount of alkylacyl analogues (1). It has been found that EPL are richer in plasmalogens than are CPL, based upon the accumulated data on levels of alkenylacyl EPL and CPL of over 60 species of marine organisms (2,3). Among these organisms, ascidian and sea urchin are eaten to some extent by native populations in Alaska and on a large scale in Asia. Recent Japanese annual landings of sea urchin gonads exceed 300 metric tons. It is now accepted that the amount of ether-linked glycerolipids in rats can be easily increased with dietary supplementation of ether-linked lipids (4–6). Therefore, ether-linked glycerophospholipids in ascidian muscle and sea urchin gonads might be incorporated into human tissues and accumulated as membrane phospholipids. However, information on the fatty chain composition of alkenylacyl and alkylacyl subclasses of marine organisms, particularly on the fatty acid composition in the *sn*-2 position of marine invertebrates, has been relatively limited (7–12). In the present study, the contents and fatty chain compositions of alkylacyl and alkenylacyl choline and EPL in muscle and viscera of an ascidian, and in sea urchin gonads, were investigated in detail, with particular emphasis on comparing the alkyl and alkenyl chain structures within specific phospholipid subclasses to investigate the possibility of these organisms as a source of dietary exogenous ether-linked lipids.

MATERIALS AND METHODS

Samples. Ascidian *Halocynthia roretzi* (16 specimens, weighing an average of 318 g) were harvested at Ishinomaki Bay, near Sendai, Japan in July 1989. The samples were separated and pooled into muscle and viscera, and were subjected to lipid analyses. Sea urchin *Strongylocentrotus intermedius* (70 specimens with an average gonad weight of *ca.* 3 g) were collected at the coast of Abashiri, Hokkaido, Japan, in December 1989. The gonads were removed, pooled, and lipids were analyzed.

Lipid extraction and fractionation. Total lipids were extracted with a mixture of chloroform/methanol, according to the Bligh and Dyer procedure (13). The respective choline and ethanolamine phospholipids (CPL and EPL, respectively) were separated by silicic acid column chromatography, according to the method of Hanahan *et al.* (14).

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Abbreviations: CPL, choline glycerophospholipids; EPL, ethanolamine glycerophospholipids; GLC, gas–liquid chromatography; NL, nonpolar lipids; PL, polar lipids; TL, total lipids; TLC, thin-layer chromatography; TMS, trimethylsilyl.

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Determination of lipid class contents. Total lipid content was determined gravimetrically. Phosphorus content in the total lipids (TL) was determined spectrophotometrically, according to Bartlett (15). The phospholipid content was obtained by multiplying the phosphorus content of the TL by 25 based on general phosphorus content in CPL. Phospholipid classes were separated by two-dimensional thin-layer chromatography (TLC) on a Silica Gel G plate (E. Merck, Darmstadt, Germany) and recovered by solvent extraction.

Analyses of alkenyl, alkyl, and acyl chain compositions. CPL and EPL fractions were each hydrolyzed with phospholipase C (*Bacillus cereus*; Boehringer Mannheim, Mannheim, Germany) to obtain the corresponding 1,2-diradyl glycerols. These were purified by preparative TLC on silicic acid and then acetylated (16). The acetylated derivatives were separated from each other by preparative TLC on Silica Gel G. The separated and purified acetylglycerol derivatives were saponified with 1 N KOH in ethanol at 85°C for 1 h. The 1-*O*-alk-1′-enylglycerols and 1-*O*-alkylglycerols thus obtained were extracted with diethyl ether and converted to their corresponding trimethylsilyl (TMS) ether derivatives, i.e., 1-*O*-alk-1′-enyl-2,3-TMS-glycerols and 1-*O*-alkyl-2,3-TMS-glycerols, respectively. The fatty acids released upon alkaline hydrolysis from the *sn*-1 and *sn*-2 positions of 1,2-diacylglycerol and from the *sn*-2 position of 1-*O*-alk-1′-enyl-2-acylglycerols and 1-*O*-alkyl-2-acylglycerols were extracted with diethyl ether and were, after methylation, analyzed by gas–liquid chromatography (GLC) under the conditions described below.

Determination of alkenylacyl, alkylacyl, and diacyl, and diacyl EPL, and CPL contents. The fatty acid contents of alkenylacyl, alkylacyl and diacyl EPL and CL in the TL were determined by GLC using 13:0 fatty acid (Sigma Chemical Company, St. Louis, MO) as an internal standard (17).

GLC and gas chromatography/mass spectrometry. The fatty chain compositions of 1-*O*-alk-1′-enyl-2,3-TMS-glycerols and 1-*O*-alkyl-2,3-TMS-glycerols derivatives were analyzed by GLC using a Shimadzu GC8APF instrument (Kyoto, Japan) equipped with a SUPELCOWAX-10 fused silica wall-coated open tubular column (30 m × 0.25 mm i.d.; Supelco Japan Ltd., Tokyo, Japan). The injector and the column were held at 250 and 210°C, respectively. Helium was used as carrier gas at a constant inlet pressure of 1.5 kg/cm^2.

TABLE 1
Lipid Contents of Ascidian and Sea Urchin Samples (wet wt%)

	Total lipids	Polar lipids	Nonpolar lipids
Ascidian *Halocynthia roretzi*			
Muscle	2.48	0.99	1.49
Viscera	3.35	0.94	2.41
Sea urchin *Strongylocentrotus intermedius*			
Gonad	4.09	1.35	2.74

Fatty acid compositions of the phospholipids were determined by GLC under similar conditions used for the analysis of the TMS ether derivatives, except that the column oven temperature was 195°C.

Mass spectrometric analysis of the TMS ether derivatives was carried out with a Shimadzu GC-MS 9020 DF instrument with a chemical ionization source to which the outlet of SUPELCOWAX-10 column (30 m × 0.25 mm i.d.) was connected directly. The column and injector temperatures were 230 and 250°C, respectively. The sample eluted from the GLC column was ionized in the chemical ionization mode by introducing *iso*-butane as a reagent gas at *ca.* 1 torr (18).

RESULTS

Lipid contents. As shown in Tables 1 and 2, the levels of TL, polar lipids (PL), and nonpolar lipids (NL) in the ascidian muscle were low. PL were predominantly composed of CPL, EPL, and sphingomyelin. There is a higher level of NL in the viscera; the level of the PL in the viscera was close to that of the muscle. The sea urchin gonad showed higher TL content compared to the ascidian samples. This is due to the higher level of both PL and NL in the sea urchin gonad than those of the ascidian samples. The sea urchin gonad PL were composed predominantly of CPL and EPL.

Contents of alkenylacyl, alkylacyl and diacyl CPL and EPL. The lipid subclass compositions of CPL and EPL are shown in Table 3. Of the ascidian muscle CPL, the diacyl subclass was the most prominent. The respective levels of alkylacyl and alkenylacyl subclasses were much lower. In the as-

TABLE 2
Lipid Class Compositions of Ascidian and Sea Urchin Samples[a]

	CPL	EPL	LysoCPL	SPM	LysoEPL	SPL	IPL	Others
Ascidian								
Muscle	49.3[b] (490)[c]	18.7 (185)	7.1 (71)	14.3 (142)	trace[d]	2.8 (28)	5.5 (54)	2.3 (23)
Viscera	48.2 (454)	25.4 (240)	1.5 (14)	11.1 (104)	trace	3.6 (34)	6.1 (58)	4.1 (39)
Sea urchin								
Gonad	63.7 (863)	18.1 (245)	trace	trace	trace	6.4 (87)	7.7 (104)	4.1 (55)

[a]CPL, choline glycerophospholipids; EPL, ethanolamine glycerophospholipids, IPL, inositol glycerophospholipids; lysoCPL, choline lysoglycerophospholipids; lysoEPL, ethanolamine lysoglycerophospholipids; SPL, serine glycerophospholipids; SPM, sphingomyelin.

[b]Values are presented as % of recovered phosphorus.

[c]Figures in parentheses show the content in mg/100 g total wet organ tissue.

TABLE 3
Contents of Alkenylacyl, Alkylacyl, and Diacyl EPL and CPL in Ascidian and Sea Urchin Samples

	Alkenylacyl		Alkylacyl		Diacyl	
	EPL	CPL	EPL	CPL	EPL	CPL
Ascidian						
Muscle	69.0[a] (128)[b]	3.1 (15)	17.2 (32)	12.5 (61)	13.8 (26)	84.4 (413)
Viscera	64.4 (154)	5.7 (26)	21.8 (52)	23.9 (109)	13.9 (33)	70.5 (320)
Sea urchin						
Gonad	67.9 (167)	4.4 (38)	5.7 (14)	46.1 (398)	26.4 (65)	49.5 (427)

[a]Figure shows the composition of subclass in EPL or in CPL. See Table 2 for abbreviations.
[b]Figures in parentheses are presented as mg/100 g of total wet organ tissues.

TABLE 4
Hydrocarbon Chain Compositions of Choline Glycerophospholipids in Ascidian Muscle[a]

Subclass	Alkenylacyl		Alkylacyl		Diacyl
Fatty chain[b] position	*sn*-1	*sn*-2	*sn*-1	*sn*-2	*sn*-1 + *sn*-2
14:0	—[c]	6.78 ± 0.18	3.96 ± 0.13	1.99 ± 0.32	10.0 ± 0.89
14:1n-5	—	—	0.44 ± 0.04	—	—
15:0 *iso*	—	2.63 ± 0.05	1.56 ± 0.05	0.81 ± 0.14	0.32 ± 0.07
15:0 *anteiso*	—	3.31 ± 0.08	—	0.99 ± 0.16	0.24 ± 0.07
15:0	—	4.22 ± 0.09	1.35 ± 0.05	1.46 ± 0.30	1.37 ± 0.08
15:1n-8	—	5.95 ± 0.25	—	1.66 ± 0.37	—
16:0 *iso*	—	3.32 ± 0.09	—	0.87 ± 0.25	0.24 ± 0.02
16:0	28.9 ± 0.76	15.3 ± 0.54	44.6 ± 0.52	4.11 ± 0.23	23.1 ± 0.32
16:1n-11	—	—	3.70 ± 0.13	—	—
16:1n-7	—	7.59 ± 0.42	—	2.24 ± 0.18	4.46 ± 0.11
16:1n-5	—	—	—	—	0.38 ± 0.01
16:2n-7	—	—	—	—	0.13 ± 0.01
16:2n-4	—	—	—	—	0.23 ± 0.01
17:0 *iso*	1.05 ± 0.12	—	0.77 ± 0.01	—	0.39 ± 0.00
17:0 *anteiso*	10.7 ± 0.66	—	0.53 ± 0.04	—	—
17:0	1.20 ± 0.04	1.13 ± 0.23	—	—	0.33 ± 0.01
17:1n-8	—	1.07 ± 0.26	—	—	0.20 ± 0.01
18:0 *iso*	3.15 ± 0.08	1.15 ± 0.62	—	—	—
18:0	26.1 ± 0.55	4.84 ± 0.25	10.7 ± 0.22	2.04 ± 0.79	2.56 ± 0.09
18:1n-9	22.9 ± 0.18	5.42 ± 0.22	26.5 ± 1.96	2.77 ± 0.12	4.62 ± 0.14
18:1n-7	—	0.85 ± 0.06	—	1.82 ± 0.05	11.3 ± 0.32
18:2n-7	2.78 ± 0.06	—	2.91 ± 0.05	—	—
18:2n-6	—	1.66 ± 0.05	—	3.31 ± 0.05	2.54 ± 0.07
18:2n-4	—	—	—	—	0.40 ± 0.01
18:3n-3	—	—	—	0.59 ± 0.04	1.16 ± 0.09
18:4n-3	—	5.48 ± 0.24	—	1.60 ± 0.02	1.22 ± 0.01
19:0	0.93 ± 0.11	—	0.40 ± 0.02	—	0.38 ± 0.01
20:0	—	—	0.30 ± 0.04	—	0.11 ± 0.01
20:1n-11	2.20 ± 0.03	—	1.04 ± 0.09	—	—
20:1n-9	—	—	—	—	0.88 ± 0.02
20:1n-7	—	—	—	—	0.85 ± 0.04
20:2 NMID (5,11)[d]	—	—	—	—	1.80 ± 0.46
20:2 NMID (5,13)	—	—	—	—	0.62 ± 0.30
20:2n-6	—	1.37 ± 0.03	—	—	0.43 ± 0.05
20:3n-6	—	—	—	—	0.29 ± 0.02
20:4n-6	—	1.78 ± 0.07	—	3.42 ± 0.01	1.02 ± 0.02
20:4n-3	—	—	—	—	0.40 ± 0.04
20:5n-3	—	19.8 ± 0.38	—	43.6 ± 0.40	18.6 ± 0.47
21:5n-3	—	—	—	5.82 ± 0.02	0.88 ± 0.01
22:5n-3	—	1.75 ± 0.26	—	0.91 ± 0.27	0.40 ± 0.03
22:6n-3	—	3.51 ± 0.44	—	20.1 ± 0.38	8.09 ± 0.29

[a]The data are presented as the mean ± standard deviation of 6 (2 groups × 3) determinations.
[b]The position of the double bond applies to fatty acyl chains.
[c]The — denotes not detected.
[d]NMID, nonmethylene-interrupted diene.

TABLE 5
Hydrocarbon Chain Compositions of Choline Glycerophospholipids in Ascidian Viscera[a]

Subclass	Alkenylacyl		Alkylacyl		Diacyl
Fatty chain[b] position	*sn*-1	*sn*-2	*sn*-1	*sn*-2	*sn*-1 + *sn*-2
14:0	—[c]	3.57 ± 0.05	4.16 ± 0.15	0.75 ± 0.01	12.2 ± 0.07
14:1n-5	—	—	0.53 ± 0.05	—	—
15:0 *iso*	—	0.57 ± 0.01	1.66 ± 0.06	—	0.88 ± 0.09
15:0 *anteiso*	—	0.56 ± 0.02	—	—	0.62 ± 0.04
15:0	—	2.47 ± 0.01	1.56 ± 0.02	0.43 ± 0.03	1.89 ± 0.07
15:1n-8	—	1.58 ± 0.01	—	—	—
16:0 *iso*	—	0.91 ± 0.02	—	—	0.56 ± 0.05
16:0	28.0 ± 0.32	23.7 ± 0.26	42.1 ± 0.61	5.53 ± 0.05	22.9 ± 0.66
16:1n-11	—	—	3.89 ± 0.07	—	—
16:1n-7	—	6.18 ± 0.08	—	1.29 ± 0.09	3.36 ± 0.25
16:1n-5	—	—	—	—	0.35 ± 0.02
16:2n-7	—	—	—	—	0.22 ± 0.02
16:2n-4	—	—	—	—	0.48 ± 0.01
17:0 *iso*	1.35 ± 0.41	—	1.97 ± 0.07	—	0.48 ± 0.04
17:0 *anteiso*	3.90 ± 0.57	—	0.87 ± 0.15	—	—
17:0	3.02 ± 0.97	0.88 ± 0.01	1.61 ± 0.28	—	0.43 ± 0.00
17:1n-8	—	1.05 ± 0.01	—	—	0.28 ± 0.01
18:0 *iso*	3.31 ± 0.30	0.38 ± 0.04	—	—	—
18:0	18.5 ± 1.00	3.62 ± 0.01	6.79 ± 0.30	0.91 ± 0.01	2.78 ± 0.07
18:1n-9	31.4 ± 1.96	6.00 ± 0.01	29.7 ± 0.33	2.05 ± 0.01	4.03 ± 0.05
18:1n-7	—	1.20 ± 0.03	—	1.64 ± 0.01	9.15 ± 0.16
18:2n-7	4.13 ± 0.11	—	3.52 ± 0.07	—	—
18:2n-6	—	2.32 ± 0.02	—	3.13 ± 0.02	2.27 ± 0.05
18:2n-4	—	—	—	0.30 ± 0.01	0.38 ± 0.01
18:3n-6	—	—	—	—	0.52 ± 0.07
18:3n-3	—	—	—	—	1.20 ± 0.07
18:4n-3	—	—	—	1.57 ± 0.26	2.06 ± 0.04
19:0	4.17 ± 0.53	—	0.35 ± 0.04	—	—
20:0	—	—	0.29 ± 0.07	—	0.17 ± 0.02
20:1n-11	2.28 ± 0.28	—	0.99 ± 0.04	—	—
20:1n-9	—	—	—	—	0.72 ± 0.02
20:1n-7	—	—	—	—	1.55 ± 0.10
20:2 NMID (5,11)[d]	—	—	—	—	0.93 ± 0.16
20:2 NMID (5,13)	—	—	—	—	0.57 ± 0.12
20:2n-6	—	—	—	—	0.42 ± 0.00
20:3n-6	—	—	—	—	0.26 ± 0.01
20:3n-3	—	—	—	0.53 ± 0.14	—
20:4n-6	—	3.62 ± 0.07	—	4.42 ± 0.05	1.12 ± 0.06
20:4n-3	—	—	—	0.33 ± 0.02	0.36 ± 0.03
20:5n-3	—	29.5 ± 0.15	—	43.7 ± 0.15	16.0 ± 0.33
21:5n-3	—	3.04 ± 0.11	—	6.77 ± 0.08	0.82 ± 0.03
22:5n-3	—	1.65 ± 0.06	—	—	0.60 ± 0.01
22:6n-3	—	7.28 ± 0.10	—	26.6 ± 0.33	9.83 ± 0.19

[a]The data are presented as the mean ± standard deviation of 6 (2 groups × 3) determinations.
[b]The position of the double bond applies to fatty acyl chains.
[c]The — denotes not detected.
[d]NMID, nonmethylene-interrupted diene.

cidian viscera CPL, the level of the diacyl subclass was lower, being offset by higher levels of alkylacyl and alkenylacyl subclasses, compared to those of the muscle CPL. Of the ascidian muscle EPL, the alkenylacyl subclass was most prominent. The levels of alkylacyl and diacyl subclasses were lower. The higher level of the alkenylacyl subclass (64.4%) was also noteworthy in the ascidian viscera sample.

In the sea urchin gonad CPL, the level of the alkylacyl subclass (46.1%) was much higher compared to the ascidian muscle and viscera CPL; however, the level of the diacyl subclass of the sea urchin gonad was lower. The level of alkenylacyl subclass was similar to that of the ascidian samples. Of the sea urchin gonad EPL, the most prominent subclass was alkenylacyl, followed by diacyl and alkylacyl subclasses in that order.

Based on the above data, it is possible to say that the most prominent lipid subclass of CPL was diacyl in the ascidian samples but was composed of both diacyl and alkylacyl subclasses in the sea urchin gonad. The alkenylacyl subclass of EPL was the most prominent in all of the samples submitted to the analyses of the present study.

Alkenyl, alkyl, and acyl chain compositions of CPL. The fatty acid and hydrocarbon chain compositions of alkenyla-

TABLE 6
Hydrocarbon Chain Compositions of Choline Glycerophospholipids in Sea Urchin Gonad[a]

Subclass	Alkenylacyl		Alkylacyl		Diacyl
Fatty chain[b] position	*sn*-1	*sn*-2	*sn*-1	*sn*-2	*sn*-1 + *sn*-2
14:0	—[c]	8.01 ± 0.17	0.63 ± 0.18	1.14 ± 0.09	1.60 ± 0.04
14:1n-5	—	4.49 ± 0.04	—	—	0.08 ± 0.00
15:0 *iso*	—	1.57 ± 0.26	—	—	0.08 ± 0.01
15:0 *anteiso*	—	2.04 ± 0.06	—	—	—
15:0	—	4.17 ± 0.23	0.13 ± 0.06	0.24 ± 0.01	0.28 ± 0.00
15:1n-8	—	7.24 ± 0.23	—	0.28 ± 0.01	0.11 ± 0.00
16:0 *iso*	—	2.12 ± 0.28	—	—	—
16:0	17.9 ± 1.01	21.3 ± 0.05	33.0 ± 0.74	2.30 ± 0.03	12.5 ± 0.07
16:1n-7	—	11.0 ± 0.88	—	2.51 ± 0.02	2.54 ± 0.09
16:1n-5	—	0.77 ± 09.32	—	0.97 ± 0.01	1.46 ± 0.00
16:2n-7	—	1.02 ± 0.45	—	—	—
17:0 *iso*	9.10 ± 1.01	—	1.06 ± 0.03	—	0.08 ± 0.00
17:0 *anteiso*	2.45 ± 0.63	—	0.25 ± 0.01	—	—
17:0	3.67 ± 0.38	1.58 ± 0.22	2.48 ± 0.66	—	0.20 ± 0.00
18:0 *iso*	—	—	0.10 ± 0.03	—	—
18:0	48.8 ± 2.92	4.92 ± 0.14	36.2 ± 0.14	0.40 ± 0.01	2.27 ± 0.01
18:1n-9	10.7 ± 0.33	8.04 ± 0.13	17.8 ± 0.22	0.83 ± 0.00	5.13 ± 0.01
18:1n-7	—	1.42 ± 0.03	—	0.41 ± 0.01	4.84 ± 0.01
18:1n-5	—	0.39 ± 0.08	—	—	0.66 ± 0.01
18:2n-7	—	—	0.35 ± 0.02	0.37 ± 0.01	0.71 ± 0.00
18:2n-6	—	1.38 ± 0.03	—	0.16 ± 0.01	0.53 ± 0.00
18:2n-4	—	0.95 ± 0.03	—	—	0.07 ± 0.00
18:3n-6	—	—	—	—	0.16 ± 0.00
18:3n-3	—	—	—	—	0.27 ± 0.01
18:3n-1	—	—	—	—	0.07 ± 0.00
18:4n-3	—	—	—	0.36 ± 0.03	0.34 ± 0.00
19:0	5.87 ± 0.59	—	0.96 ± 0.08	—	—
19:1n-8	—	—	—	—	0.53 ± 0.00
20:0	1.50 ± 0.17	0.29 ± 0.02	1.38 ± 0.12	—	0.27 ± 0.00
20:1n-15	—	—	5.00 ± 0.45	—	7.39 ± 0.01
20:1n-9	—	—	—	—	3.88 ± 0.12
20:1n-7	—	—	—	—	0.81 ± 0.01
20:2 NMID (5,11)[d]	—	1.67 ± 0.05	0.64 ± 0.05	0.93 ± 0.01	11.4 ± 0.03
20:2 NMID (5,13)	—	0.33 ± 0.02	—	0.52 ± 0.01	4.35 ± 0.06
20:2n-6	—	—	—	—	1.57 ± 0.02
20:3n-6	—	—	—	—	0.61 ± 0.02
20:3n-3	—	—	—	—	1.14 ± 0.04
20:4n-6	—	7.08 ± 0.27	—	30.1 ± 0.06	12.8 ± 0.05
20:5n-3	—	8.23 ± 0.25	—	54.9 ± 0.08	17.5 ± 0.09
22:0	—	—	—	—	0.28 ± 0.02
22:1n-9	—	—	—	—	0.28 ± 0.01
22:2 NMID (7,13)	—	—	—	—	0.10 ± 0.01
20:2 NMID (7,15)	—	—	—	—	0.44 ± 0.01
22:5n-3	—	—	—	—	0.37 ± 0.01
Unknown	—	—	—	2.59 ± 0.03	3.24 ± 0.02

[a]The data are presented as the mean ± standard deviation of 6 (2 groups × 3) determinations.
[b]The position of the double bond applies to fatty acyl chains.
[c]The — denotes not detected.
[d]NMID, nonmethylene-interrupted diene.

cyl, alkylacyl, and diacyl subclasses of the CPL in the ascidian muscle are shown in Table 4. 16:0, 18:0, and 18:1 alkenyl chains were prominent. The high level of 17:0 *anteiso* (10.7%) was unexpected. Although the prominent fatty acids in the *sn*-2 position of the alkenylacyl CPL were 20:5n-3 and 16:0, the levels of other acids were low, ranging from 1 to 7%.

The 16:0, 18:1, and 18:0 alkyl chains were prominent. Shorter alkyl chains, i.e., 14:0 and 15:0, were recognized. By contrast, the corresponding shorter alkenyl chains were not detected. The 20:5n-3 and 22:6n-3 fatty acids were prominent in the alkylacyl CPL.

The fatty acid and hydrocarbon chain compositions of alkenylacyl, alkylacyl, and diacyl subclasses of the CPL in the ascidian viscera are shown in Table 5. The compositions of the other alkenyl and alkyl chains were almost identical to those in the muscle CPL; however, the levels of the odd-numbered alkenyl chains, i.e., 17:0 and 19:0, were higher.

The fatty acid and hydrocarbon chain compositions of alkenylacyl, alkylacyl, and diacyl subclasses of the sea urchin gonad CPL are summarized in Table 6. The alkenyl chain

TABLE 7
Hydrocarbon Chain Compositions of Choline Glycerophospholipids in Ascidian Muscle[a]

Subclass	Alkenylacyl		Alkylacyl		Diacyl
Fatty chain[b] position	*sn*-1	*sn*-2	*sn*-1	*sn*-2	*sn*-1 + *sn*-2
14:0	—[c]	0.70 ± 0.27	2.18 ± 0.14	1.84 ± 0.64	1.81 ± 0.49
14:1n-5	—	—	0.64 ± 0.07	—	—
15:0 *iso*	—	—	1.00 ± 0.07	0.82 ± 0.29	0.52 ± 0.19
15:0 *anteiso*	—	—	—	0.46 ± 0.22	0.53 ± 0.22
15:0	—	0.51 ± 0.14	0.60 ± 0.03	1.03 ± 0.27	0.91 ± 0.29
15:1n-8	—	0.45 ± 0.15	—	1.62 ± 0.24	1.05 ± 0.21
16:0 *iso*	—	—	—	0.49 ± 0.13	0.37 ± 0.16
16:0	10.0 ± 0.24	1.79 ± 0.16	34.4 ± 0.89	5.75 ± 0.12	12.8 ± 0.24
16:1n-11	—	—	2.01 ± 0.20	—	—
16:1n-7	—	0.70 ± 0.32	—	1.73 ± 0.25	1.73 ± 0.25
16:1n-5	—	0.21 ± 0.03	—	0.33 ± 0.08	0.33 ± 0.19
16:2n-4	—	—	—	—	0.50 ± 0.03
17:0 *iso*	3.11 ± 0.06	0.44 ± 0.03	1.40 ± 0.06	1.31 ± 0.06	0.72 ± 0.27
17:0 *anteiso*	0.62 ± 0.01	—	1.10 ± 0.03	—	—
17:0	2.90 ± 0.02	0.87 ± 0.10	1.56 ± 0.03	0.58 ± 0.02	0.93 ± 0.07
17:1n-8	—	—	—	—	0.14 ± 0.01
18:0 *iso*	1.21 ± 0.04	—	—	—	0.32 ± 0.02
18:0	69.4 ± 0.44	0.63 ± 0.02	24.6 ± 0.81	2.01 ± 0.11	28.5 ± 0.49
18:1n-9	8.54 ± 0.10	3.48 ± 0.11	22.6 ± 0.36	2.19 ± 0.10	3.82 ± 0.08
18:1n-7	—	0.22 ± 0.00	—	0.72 ± 0.05	2.75 ± 0.06
18:2n-7	0.51 ± 0.01	—	2.31 ± 0.05	—	—
18:2n-6	—	0.29 ± 0.02	—	0.53 ± 0.06	0.72 ± 0.03
18:3n-3	—	—	—	—	0.32 ± 0.00
18:4n-3	—	0.84 ± 0.03	—	0.70 ± 0.12	—
19:0	1.24 ± 0.05	—	0.77 ± 0.12	—	1.48 ± 0.04
20:0	0.20 ± 0.06	—	0.41 ± 0.03	—	0.62 ± 0.02
20:1n-11	2.19 ± 0.08	—	2.42 ± 0.26	—	0.63 ± 0.05
20:1n-9	—	—	—	—	0.31 ± 0.02
20:1n-7	—	—	—	—	1.99 ± 0.05
20:2 NMID (5,13)[d]	—	—	—	—	0.71 ± 0.00
20:3n-3	—	—	—	1.17 ± 0.02	0.65 ± 0.02
20:4n-6	—	7.64 ± 0.04	—	3.42 ± 0.05	0.73 ± 0.04
20:5n-3	—	64.5 ± 0.59	—	55.9 ± 0.85	10.3 ± 0.17
21:5n-3	—	2.61 ± 0.12	—	5.21 ± 0.30	0.91 ± 0.03
22:6n-3	—	14.1 ± 0.59	—	13.2 ± 0.19	21.3 ± 0.23

[a]The data are presented as the mean ± standard deviation of 6 (2 groups × 3) determinations.
[b]The position of the double bond applies to fatty acyl chains.
[c]The — denotes not detected.
[d]NMID, nonmethylene-interrupted diene.

compositions were quite simple; a subtotal of 18:0 and 16:0 alkenyl chains amounted to 66.7%. An unexpected amount of *iso* 17:0 alkenyl chain was included. The levels of polyunsaturated fatty acids, such as 20:5n-3 and 20:4n-6, were low among the fatty acids of the *sn*-2 position of the alkenylacyl subclass.

The alkyl chain composition was also simple; 18:0, 16:0, and 18:1 alkyl chains were prominent. By contrast, the levels of shorter alkyl chains, such as 16:0 and 14:0, were higher than those of the alkenyl chains with the same carbon chains. The levels of 20:4n-6 and 20:5n-3 in the *sn*-2 position were extremely high.

Alkenyl, alkyl, and acyl chain compositions of EPL. The fatty acid and hydrocarbon chain compositions of alkenylacyl, alkylacyl, and diacyl subclasses of EPL in the ascidian muscle are shown in Table 7. The 18:0, 16:0, and 18:1 alkenyl hydrocarbon chains were predominant. Among the fatty acids, 20:5n-3, 22:6n-3, and 20:4n-6 were prominent.

The 16:0, 18:0, and 18:1 alkyl chains were prominent. The 20:5n-3 and 22:6n-3 fatty acyl chains were linked selectively to the *sn*-2 position of the alkylacyl CPL.

Table 8 summarizes the fatty acid and hydrocarbon chain compositions of alkenylacyl, alkylacyl, and diacyl subclasses of the ascidian viscera EPL. The compositions of both the alkenyl chains and alkyl chains of the viscera EPL and of the muscle EPL closely resembled each other. In the acyl chain compositions of the alkenylacyl and alkylacyl subclasses, however, the levels of 20:5n-3 and 22:6n-3 of the viscera sample were lower than those of the muscle sample.

The fatty acid and hydrocarbon chain compositions of alkenylacyl, alkylacyl, and diacyl subclasses of EPL in the sea urchin gonad are summarized in Table 9. Although the locations of ethylenic bond in the alkenyl and acyl chains were not identified under the experimental conditions used in the present study, it is possible to separate the positional isomers from each other by GLC (8). Mass spectrometric data (not

TABLE 8
Hydrocarbon Chain Compositions of Choline Glycerophospholipids in Ascidian Viscera[a]

Subclass	Alkenylacyl		Alkylacyl		Diacyl
Fatty chain[b] position	*sn*-1	*sn*-2	*sn*-1	*sn*-2	*sn*-1 + *sn*-2
14:0	—[c]	1.13 ± 0.21	2.11 ± 0.12	3.07 ± 0.56	2.83 ± 0.44
14:1n-5	—	—	1.60 ± 0.08	—	—
15:0 *iso*	—	—	0.88 ± 0.04	—	—
15:0 *anteiso*	—	0.37 ± 0.14	—	1.07 ± 0.37	0.68 ± 0.21
15:0	—	0.83 ± 0.22	0.57 ± 0.02	2.09 ± 0.47	1.68 ± 0.28
15:1n-8	—	0.75 ± 0.28	—	1.77 ± 0.40	1.31 ± 0.22
16:0 *iso*	—	0.47 ± 0.15	—	1.07 ± 0.23	0.83 ± 0.19
16:0	9.45 ± 0.16	4.85 ± 0.04	29.5 ± 0.73	13.2 ± 0.38	14.1 ± 0.33
16:1n-11	—	—	1.32 ± 0.07	—	—
16:1n-7	—	1.88 ± 0.18	—	4.53 ± 0.07	3.24 ± 0.13
16:1n-5	—	0.31 ± 0.09	—	0.49 ± 0.15	0.25 ± 0.08
16:2n-4	—	0.94 ± 0.08	—	0.58 ± 0.07	0.44 ± 0.02
17:0 *iso*	3.49 ± 0.03	0.62 ± 0.03	4.66 ± 0.08	0.56 ± 0.12	1.00 ± 0.19
17:0 *anteiso*	0.84 ± 0.05	—	0.90 ± 0.04	—	—
17:0	3.22 ± 0.04	0.85 ± 0.08	1.69 ± 0.08	1.28 ± 0.02	1.94 ± 0.02
17:1n-8	—	—	—	0.60 ± 0.12	0.51 ± 0.10
18:0 *iso*	1.26 ± 0.15	—	—	—	0.43 ± 0.03
18:0	70.3 ± 0.86	0.81 ± 0.02	29.0 ± 0.24	1.97 ± 0.04	20.1 ± 0.26
18:1n-9	7.34 ± 0.11	2.96 ± 0.10	21.2 ± 0.23	4.46 ± 0.14	4.71 ± 0.08
18:1n-7	—	0.26 ± 0.09	—	0.74 ± 0.13	3.85 ± 0.11
18:2n-7	0.57 ± 0.21	—	2.33 ± 0.31	—	—
18:2n-6	—	0.30 ± 0.00	—	0.66 ± 0.04	0.74 ± 0.08
18:2n-4	—	—	—	0.26 ± 0.02	0.42 ± 0.16
18:3n-3	—	—	—	—	1.82 ± 0.10
18:4n-3	—	0.57 ± 0.02	—	0.52 ± 0.10	0.85 ± 0.11
19:0	2.01 ± 0.29	—	1.05 ± 0.08	—	1.04 ± 0.02
20:0	—	—	0.59 ± 0.22	—	1.03 ± 0.05
20:1n-11	1.53 ± 0.23	—	2.68 ± 0.34	—	—
20:1n-9	—	—	—	—	0.94 ± 0.26
20:1n-7	—	—	—	—	2.80 ± 0.21
20:2 NMID (5,13)[d]	—	—	—	—	0.73 ± 0.14
20:3n-3	—	0.35 ± 0.04	—	0.66 ± 0.21	0.68 ± 0.03
20:4n-6	—	7.66 ± 0.18	—	3.77 ± 0.14	1.92 ± 0.09
20:5n-3	—	51.7 ± 0.53	—	38.8 ± 1.31	10.2 ± 0.22
21:5n-3	—	3.50 ± 0.10	—	5.01 ± 0.19	1.40 ± 0.01
22:5n-3	—	0.85 ± 0.06	—	0.82 ± 0.21	—
22:6n-3	—	18.1 ± 0.51	—	12.2 ± 0.23	13.3 ± 0.40

[a]The data are presented as the mean ± standard deviation of 6 (2 groups × 3) determinations.
[b]The position of the double bond applies to fatty acyl chains.
[c]The — denotes not detected.
[d]NMID, nonmethylene-interrupted diene.

shown) proved that these separated components on GLC were certain positional isomers of alkenyl and alkyl hydrocarbon chains: that is, 18:1 and 20:1 alkenyl chains and 18:1 and 20:1 alkyl chains.

DISCUSSION

Rapport and Alonzo (19) reported that 40–50% of EPL and 20–30% of CPL were plasmalogens in several marine invertebrates. More recent systematic survey of plasmalogen for more than 60 species of marine invertebrates established that these marine organisms are rich sources of plasmalogens (19.4 to 92.5% in the EPL fraction), and that EPL are richer in the levels of plasmalogens than are CPL (2). The level of the alkenylacyl glycerophospholipids of the ascidian muscle analyzed in the present study was lower than that reported previously, in which the alkenylacyl glycerophospholipids accounted for 74.8% of EPL (3). The levels of alkylacyl glycerophospholipids of the sea urchin gonad examined in this study were, however, higher compared to the results reported previously (2).

Vysotskii *et al.* (12) reported the composition of dimethylacetal released from the PL of ascidian. They found 18:0 (75.7%) and 16:0 (7.7%) as prominent dimethylacetals. Roots and Johnston (20) found that the major fatty aldehydes released from EPL of goldfish brain were 18:0, 18:1, and 16:0 alkenyl chains, and that from CPL was 16:0 alkenyl chain. Chacko *et al.* (21) found the major aldehyde composition of the garfish olfactory nerve EPL consisted of 18:1 (65.1%), 18:0 (18.0%), and 16:0 (8.8%) alkenyl chains. The major aldehydes released from CPL were 16:0 (56.1%), 18:1 (20.9%), and 18:0 (9.4%). Snyder *et al.* (22) analyzed phos-

TABLE 9
Hydrocarbon Chain Compositions of Ethanolamine Glycerophospholipids in Sea Urchin Gonad[a]

Subclass	Alkenylacyl		Alkylacyl		Diacyl
Fatty chain[b] position	*sn*-1	*sn*-2	*sn*-1	*sn*-2	*sn*-1 + *sn*-2
14:0	—[c]	0.43 ± 0.04	—	4.09 ± 1.30	0.87 ± 0.06
14:1n-5	—	—	—	1.23 ± 0.42	—
15:0 *iso*	—	0.11 ± 0.00	—	1.90 ± 0.36	0.27 ± 0.00
15:0 *anteiso*	—	—	—	0.90 ± 0.44	0.22 ± 0.01
15:0	—	0.17 ± 0.02	—	2.71 ± 0.31	0.60 ± 0.02
15:1n-8	—	0.24 ± 0.01	—	4.15 ± 0.73	0.39 ± 0.02
16:0 *iso*	—	—	—	1.28 ± 0.41	0.20 ± 0.02
16:0	7.31 ± 0.39	1.41 ± 0.02	17.5 ± 0.34	11.6 ± 0.37	3.71 ± 0.04
16:1n-7	—	0.51 ± 0.02	—	4.28 ± 0.17	0.82 ± 0.04
16:1n-5	—	—	—	—	0.14 ± 0.02
17:0 *iso*	1.05 ± 0.06	—	5.19 ± 0.67	—	—
17:0 *anteiso*	0.32 ± 0.05	—	1.21 ± 0.32	—	—
17:0	2.44 ± 0.20	0.11 ± 0.00	3.57 ± 0.32	0.89 ± 0.08	0.20 ± 0.00
18:0 *iso*	0.48 ± 0.01	—	—	—	—
18:0 *anteiso*	0.15 ± 0.00	—	—	—	—
18:0	60.2 ± 1.07	0.44 ± 0.03	37.7 ± 1.20	3.22 ± 0.19	9.86 ± 0.07
18:1n-9	11.4 ± 0.48	0.56 ± 0.01	16.2 ± 0.54	4.30 ± 0.24	2.14 ± 0.02
18:1n-7	—	—	—	0.98 ± 0.15	1.61 ± 0.01
18:2n-7	0.65 ± 0.31	—	—	—	—
18:2n-6	—	—	—	0.80 ± 0.05	0.13 ± 0.00
18:2n-4	—	0.10 ± 0.01	—	1.27 ± 0.06	0.21 ± 0.07
19:0	1.03 ± 0.0	—	—	—	0.20 ± 0.04
19:1n-8	—	—	—	—	1.09 ± 0.02
20:0	1.85 ± 0.07	—	3.27 ± 0.11	—	0.46 ± 0.05
20:1n-15	12.1 ± 0.51	—	14.2 ± 0.46	—	26.2 ± 0.12
20:1n-9	—	—	—	—	3.11 ± 0.02
20:1n-7	—	—	—	—	0.46 ± 0.02
20:2 NMID (5,11)[d]	1.20 ± 0.03	7.07 ± 0.01	1.17 ± 0.04	0.91 ± 0.11	5.21 ± 0.03
20:2 NMID (5,13)	—	0.91 ± 0.01	—	—	2.05 ± 0.02
20:2n-6	—	0.11 ± 0.02	—	—	0.52 ± 0.02
20:3n-3	—	—	—	—	0.47 ± 0.01
20:4n-6	—	42.9 ± 0.05	—	12.9 ± 0.73	19.4 ± 0.08
20:5n-3	—	42.5 ± 0.07	—	42.5 ± 2.26	16.0 ± 0.04
22:0	—	0.46 ± 0.04	—	—	—
22:2 NMID (7,13)	—	—	—	—	1.16 ± 0.17
Unknown	—	2.02 ± 0.06	—	—	2.41 ± 0.02

[a]The data are presented as the mean ± standard deviation of 6 (2 groups × 3) determinations.
[b]The position of the double bond applies to fatty acyl chains.
[c]The — denotes not detected.
[d]NMID, nonmethylene-interrupted diene.

pholipids of the starfish digestive reticulum and found that the major aldehydes of phospholipids were 18:0 (39%), 16:0 (31%), and 20:1 (13%). In our previous investigation on the bonito white muscle CPL (18), the alkyl and alkenyl chain compositions were 16:0 (55.5%) and 14:0 (11.5%), and 16:0 (75.4%) and 18:1 (4.0%), respectively. For the EPL of the bonito sample, 16:0 (47.8%), 18:0 (15.1%), and 18:1 isomers (10.8 and 6.0%) alkyl chains, and 16:0 (53.3%), 18:0 (19.4%), and 18:1 isomers (10.8 and 3.9%) alkenyl chains were prediminent. In the Japanese oyster wet tissues (8), the most prominent alkenyl and alkyl chains of CPL were 18:0 (63.3%) and 16:0 (44.0%), respectively. The most prominent alkenyl and alkyl chains of EPL were 18:0 (64.7%) and 18:0 (51.5%), respectively.

There have been few published studies on distribution of alkylacyl glycerophospholipids compared to those of alkenylacyl subclass. It is now accepted, however, that very significant amounts of alkylacyl glycerophospholipids exist in those marine organisms with high plasmalogen contents (21,23–25). This is believed, in the case of mammalian tissues, to be due to the biosynthesis of plasmalogens; alkylacyl subclass once formed can be converted directly to the corresponding alkenylacyl subclass by a certain enzyme system present in the microsomal fraction (26–28). Thompson (29) investigated the incorporation of ^{14}C-labeled feeds into phospholipids of the terrestrial slug *Arion ater* and concluded that the alkyl chain is converted to the alkenyl chain without cleavage of the ether bond. Other major studies *in vivo* with the tissue of tumor cells (30), rat brain (31), and a slime mold (32) revealed that alkenylacyl subclasses are produced by a direct α,β-desaturation of the corresponding alkylacyl subclasses in the biosynthesis of phospholipids. In our study, however, we did not find strong similarities between the alkyl and alkenyl chains within the ether lipid types isolated from

two different marine invertebrates. This is probably due to the differences in the rate of catabolism of alkenylacyl subclass; in the case of rat liver (33), a microsomal enzyme hydrolyzes the ether bond of lyso CPL plasmalogen but not that of intact CPL plasmalogen. In mammals, it is known that the 1′-alkenyl chain lengths are restricted to a narrow range by the substrate specificity of the acyl-CoA reductase (34) and also by the chain length specificity of the dehydrogenase which catalyzes conversion of 1-*O*-alkyl glycerophospholipids to 1-*O*-alk-1′-enyl glycerophospholipids (35).

Although the biological and physiological roles of alkenylacyl and alkylacyl glycerophospholipids still remain unresolved, almost complete absence of ether lipids in the tissues of patients suffering from Zellweger cerebrohepatorenal syndrome (36) is observed. It is now accepted that dietary alkyl glyceryl ethers are not only absorbed and converted to plasmalogens in intestinal tissues, but also transported to other organs where they are metabolized and deposited as membrane plasmalogens (37). The present results revealed that ascidian muscle and sea urchin gonads are rich sources of ether-linked glycerophospholipids and they might play a role as a source of the exogenous ether lipids for these patients (6).

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Dietary Modulation of Phospholipid Fatty Acid Composition and Lipoxygenase Products in Mouse Lung Homogenates

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ABSTRACT: This study investigated the potential of dietary fats to modulate the arachidonic acid content of mouse lung phospholipids and the formation of lipoxygenase products from arachidonic and eicosapentaenoic acids. Prior to breeding, female mice were fed for five months diets with 10 wt% of either olive oil, safflower oil, fish oil, or linseed oil. The same diets were fed to the females during gestation and to the pups from day 18 to day 42 postpartum. On day 42, the phospholipids were extracted from fresh lung tissue and separated into classes [phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), and phosphatidylinositol (PI)] by thin-layer chromatography. Methyl esters of phospholipid fatty acids and unesterified fatty acids were analyzed by gas chromatography. At comparable dietary n-3/n-6 ratios, arachidonic acid was reduced 85 and 75% in lungs from mice fed linseed oil and fish oil, respectively, compared to lungs of safflower oil-fed mice. Dietary fats affected the proportion of arachidonic acid in phospholipids in the order: PE > PC > PS > PI. Following incubation of homogenized lung tissue, the total amount of 12-lipoxygenase products was lowest in lungs from mice fed olive oil, and 12-hydroxyeicosatetraenoic acid was lowest in incubated lungs from mice fed linseed oil. Comparison of the amounts of lipoxygenase substrate fatty acids in the individual phospholipids with the lipoxygenase products suggested that the major substrate pool for the 12-lipoxygenase pathway in mouse lung homogenates was PC.

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The oxygenated metabolites synthesized from arachidonic acid are important mediators in a variety of biological processes (1). The suggestion has been made that excessive biosynthesis of these compounds is associated with physiological dysfunctions, such as thrombosis (2), cancer metastasis (3), inflammatory disease, asthma (4), psoriasis (5), and autoimmune diseases, such as rheumatoid arthritis (6). Lipoxygenases are enzymes that convert arachidonic acid into biologically-active compounds, such as leukotrienes, lipoxins, and an array of hydroxyl derivatives (1). In mammalian lungs, 12- and 15-lipoxygenases catalyze reactions that produce 12-hydroxyeicosatetraenoic (12-HETE) and 15-hydroxyeicosatetraenoic acid (15-HETE) from arachidonic acid (7,8). While the functions of some 5-lipoxygenase products have been well established, the physiological roles of the 12- and 15-lipoxygenases are not clearly understood. Although the association of several biological functions with 12- and 15-lipoxygenase pathways has been suggested (9), the involvement of the 12-lipoxygenase product 12-HETE in tumor cell metastasis is most striking (10). Studies have shown that 12-HETE promotes tumor-cell adhesion by increasing surface expression of alpha-v-beta-3 integrins on endothelial cells (11). Recently, a specific receptor for 12-HETE in Lewis-lung carcinoma cells was reported (12). Also, dietary maneuvers that enhanced arachidonic acid levels were associated with increased metastasis to the lung (13,14). These findings underline the potential importance of 12-lipoxygenase products in cellular activities, particularly in promoting the adhesion of tumor cells (10). Thus, it would be important to understand how the production of 12-lipoxygenase metabolites is regulated *in vivo*.

Considerable research has focused on understanding how dietary fatty acids are incorporated into cell membrane lipids and how this affects cell function. Changes in the fatty acid composition of tissue membrane lipids are routinely seen in response to dietary changes. Furthermore, biochemical studies show that specific changes in the fatty acyl composition of membrane phospholipids alter cellular and physiological processes, such as platelet aggregation, leukocyte chemotaxis, macrophage cytolysis, and tumor metastasis (15–17). Since these processes are frequently unbalanced in disease states, manipulation of membrane composition could result in important clinical benefits. Manipulation of fatty acids in membranes also alters synthesis of their oxygenated derivatives. Since different fatty acid oxygenation pathways show varying sensitivity to nutritional modulation, it is of interest to investigate how 12-lipoxygenase activity is regulated by dietary polyunsaturated fatty acids (PUFA).

Of particular interest are the n-3 family of PUFA and how they modulate tissue lipid composition, arachidonic acid metabolism, and eicosanoid production. Dietary supplementation with fish oil has been suggested to be beneficial in some

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Abbreviations: 12-HETE, 12-hydroxyeicosatetraenoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids.

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disease states that involve arachidonic acid metabolism (18). Although the mechanism by which n-3 PUFA in fish oil exert their effects is not completely understood, the putative beneficial effects may be derived from either modification of the arachidonic acid pool through n-3 PUFA replacement or the competition of n-3 PUFA-derived oxygenated metabolites (19,20). However, data are lacking to evaluate the ability of shorter-chain n-3 PUFA to modify arachidonic acid content in lung membrane phospholipids and to alter eicosanoid production.

The present study investigated differences in metabolism and incorporation of n-3 PUFA from two sources, linseed oil, which is rich in 18:3n-3, and menhaden fish oil, which is rich in 20:5n-3 and 22:6n-3, in comparison with oils rich in n-6 PUFA (safflower oil) and n-9 monounsaturates (olive oil). Previous studies with diets containing low n-3/n-6 ratios found that 18:3n-3 is a relatively inferior source in respect to the net accumulation of 20:5n-3 in tissues and the modulation of eicosanoid synthesis (21). In the present study, unsaturated fats were fed as both 18:3n-3, and 20:5n-3 plus 22:6n-3, at high n-3/n-6 ratio during the developmental period of mice. The mouse lung was chosen because: (i) previous work documents the differential incorporation of 18:3n-3 and fish oil n-3 PUFA (22), (ii) large quantities of this tissue are available for lipid analysis, and (iii) the organ releases a variety of lipid-derived bioactive compounds. The lung may be a good model for the study of lipoxygenase activities and functions (7,9,23).

MATERIALS AND METHODS

Animal and dietary protocols. Experimental animals were 42-day-old, pathogen-free Swiss Webster mice (Bantin & Kingman, Fremont, CA). The initial treatment, grouping, diet protocol, and breeding schemes of dams were previously described in detail (22). In brief, dams were divided into four groups and fed a nonpurified diet (Rodent Laboratory Chow no. 5001; Purina Mills, St. Louis, MO) for two weeks. The groups of mice were then fed one of four synthetic diets for a five-month period. Litters from each group were culled to ten pups/litter on day 3 postpartum. On day 18, the dams were removed from the cages. The pups were fed the synthetic diet continuously until day 42, when they were killed by CO_2 intoxication. Lipid analysis and eicosanoid production assays were conducted on lungs of the 42-day-old pups.

Synthetic diets consisted of fat-free AIN-76A powdered diets (Dyets, Bethlehem, PA) modified by addition of the following dietary fats at 10 g/100 g: safflower oil (Dyets), linseed oil (Spectrum Naturals, Petaluma, CA), olive oil (G. Sensat, Extra Virgin no. 5; Specialty Food and Beverage Sales, West Milford, NJ), or a 9:1 (w/w) mixture of menhaden oil (NIH Biomedical Test Materials Program, Bethesda, MD) and safflower oil. Safflower oil was added to the fish oil diet to ensure that the linoleic acid requirement was met and to achieve a 3:1 ratio of n-3/n-6. The fatty acid composition of each oil and ratios of n-3/n-6 are summarized in Table 1. Tocopherol (0.02 g/100 g oil) and *t*-butyl-hydroquinone (0.02 g/100 g oil) levels were constant across all of the dietary groups.

TABLE 1
Fatty Acid Composition of Oils Added to Experimental Diets[a]

Fatty acid	Safflower oil	Olive oil	Linseed oil	Fish oil
16:0	7[b]	14	6	16
18:0	2	3	4	3
18:1n-9	12	74	17	10
18:2n-6	78	7	16	7
18:3n-3	—[c]	0.6	57	1
20:5n-3	—	—	—	14
22:6n-3	—	—	—	12
n-3/n-6	<0.1	0.1	3	3

[a]Diets contained 10 wt% of either safflower oil, olive oil, linseed oil, or fish/safflower at 9:1 (w/w) ratio.
[b]Only key fatty acids are shown. Values are means of two determinations per oil and are expressed as mol%.
[c]Fatty acid concentrations were below 0.01 mol% of total fat.

Tissue lipid analysis. Tissue lipids were extracted in the presence of sodium dodecyl sulfate (24) with >90% recovery of total phospholipids. They were separated into classes by thin-layer chromatography on 10 × 10 cm pre-coated silica gel 60 plates (E. Merck, Darmstadt, Germany) developed in a solvent system consisting of chloroform/methanol/acetic acid/water (50.0:37.5:3.5:2.0, by vol) (25). Individual lipid classes were visualized with 8-hydroxyl-1,3,6-pyrenetrisulfonic acid trisodium salt (Eastman Kodak Co., Rochester, NY) (26). The bands containing phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylethanolamine (PE) were scraped, and the fatty acids were directly transesterified to methyl esters with 3 N methanolic HCl (Supelco, Bellefonte, PA) at 60°C overnight. Dipentadecanoyl PC was used as an internal standard to quantify recovery and yields and was added into each sample vial prior to methylation. Fatty acid methyl esters were extracted, and fatty acid composition of each phospholipid class was determined by gas chromatography on a Hewlett-Packard model 5890 gas chromatograph (Hewlett-Packard Co., Palo Alto, CA) equipped with a flame-ionization detector and a DB-23 25 m × 0.25 mm capillary column (J&W Scientific, Folsom, CA). Fatty acid methyl esters were identified by their retention time relative to known standards (Nu-Chek-Prep, Elysian, MN).

Unesterified fatty acids in fresh lung homogenates were extracted after 10 min incubation at 37°C in the same buffer as described above, in the presence of the 12-lipoxygenase specific inhibitor esculetin (10 μM) (27), methylated with ethereal diazomethane (27), separated from other lipid classes using solid-phase extraction columns (28) (Analytichem International, Harbor City, CA), and analyzed by gas chromatography as described (21,29).

Eicosanoid synthesis by lung tissue. To determine the effect of dietary modifications on the biosynthesis of eicosanoids, the lipoxygenase products generated by homogenized lung tissue were characterized. Mice were anesthetized and

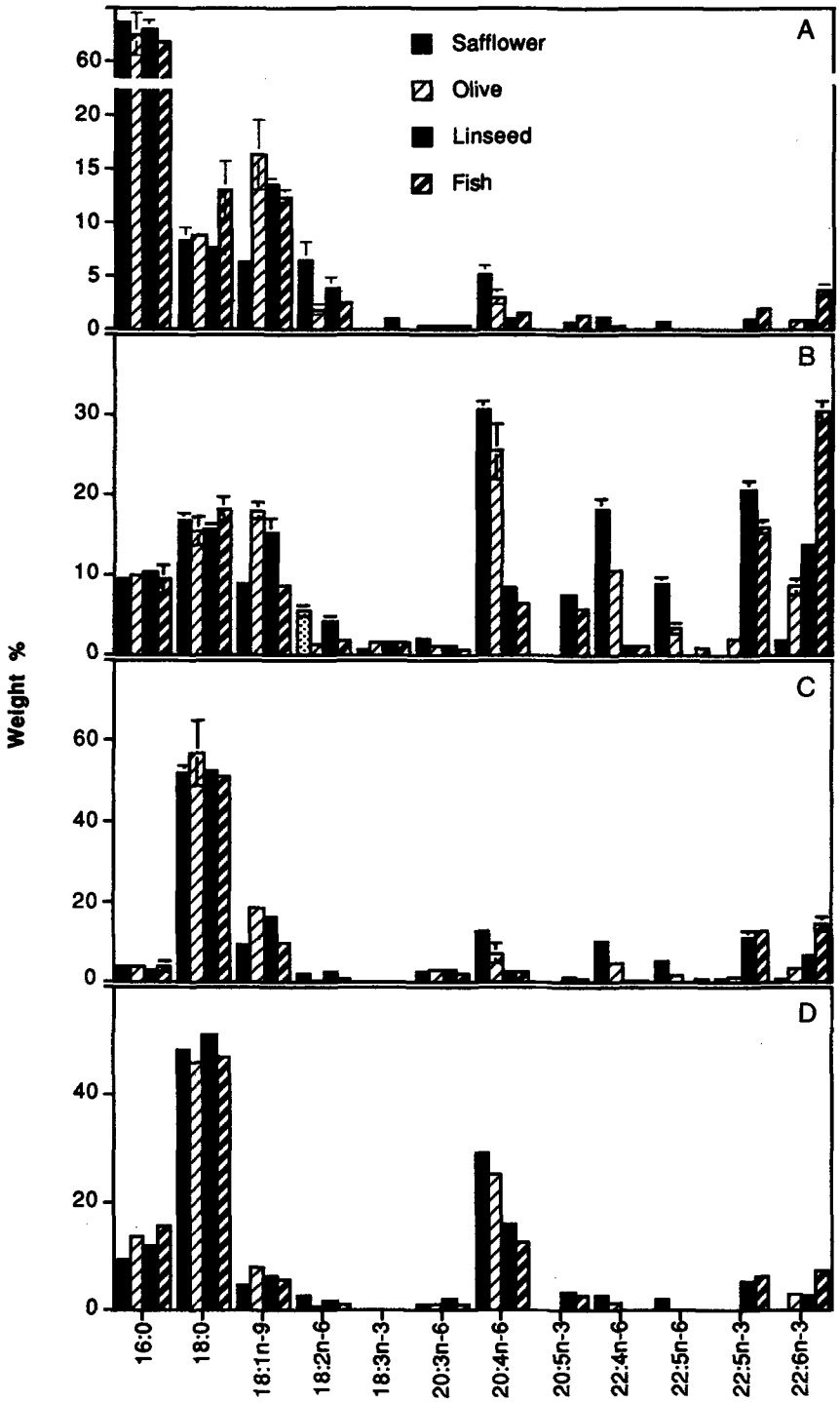


FIG. 1. Composition of major fatty acids in murine lung phospholipids after dietary fat modulation. Groups of animals were fed diets containing either safflower oil, olive oil, linseed oil, or fish oil. Values (n = 4) are expressed as mol%. A) phosphatidylcholine; B) phosphatidylethanolamine; C) phosphatidylserine; and D) phosphatidylinositol.

perfused with saline. The lungs were removed, rinsed with cold 0.05 M potassium phosphate buffer (pH 7.8), a buffer shown previously to be effective in assaying lipoxygenase activities, and homogenized in 5 vol of the same buffer. One-milliliter aliquots of the lung homogenates were incubated in a shaker-water bath at 37°C for 30 min. After incubation, the assay mixtures were acidified with 0.2 M acetic acid (pH 4.0) and lipid metabolites were extracted twice with 2 mL ethyl acetate. The extracts were evaporated to dryness under nitrogen, and lipoxygenase products were methylated with 200 μL

ethereal diazomethane. The methylated products were then separated and purified using silica solid-phase columns (Analytichem International) as previously described (28).

The analysis of hydroxy fatty acid methyl esters was carried out by reverse-phase high-pressure liquid chromatography using a Hewlett-Packard model 1090 high pressure liquid chromatograph with a 25 cm × 4.6 mm DB C-18 column (Supelco, Bellefonte, PA). The products were eluted isocratically using 75% methanol and 25% of a mixture of 5 mM ammonium acetate and 30% methanol containing 1 mM EDTA. The absorbance of eluted compounds at 234 nm was automatically recorded using a diode array detector (model 1040; Hewlett Packard, Bellefonte, PA). Lipoxygenase products were identified by comparing the retention times and/or ultraviolet spectra with those of authentic standards (7).

Statistical analysis. Data were analyzed using the *t*-test and analysis of variance (Microsoft® Excel; Microsoft Corporation, Redmond, WA).

RESULTS

Fatty acid composition of lung phospholipids. Figure 1 summarizes the fatty acid composition of individual phospholipids from the lungs of groups of mice fed weaning diets with either safflower oil, olive oil, linseed oil, or fish oil; 16:0 represented approximately 80% of the total fatty acids in the PC fraction from lungs of all dietary groups. Among the dietary groups, there were no differences in the amount of 16:0 in any of the individual phospholipid classes in spite of the highly varied intakes of long-chain PUFA during growth and development of the animals. Overall, for each of the dietary groups, the sum of saturated fatty acids was similar in PC, PS, PI, and PE; however, the proportions of C_{20} plus C_{22} unsaturated fatty acids were higher in the lung total phospholipids of mice fed safflower oil and fish oil than in those from mice fed linseed oil or olive oil ($P < 0.05$).

Arachidonic acid in lung PC in the safflower oil-fed mice (5.1%) was significantly different ($P < 0.05$) from that in the linseed oil-mice (1.0%), as shown in Figure 1A. Interestingly, the lower percentage of arachidonic acid in PC was accompanied by higher percentages of oleic acid (18:1n-9), eicosapentaenoic acid (20:5n-3), and docosahexaenoic acid (22:6n-3).

PE accumulated a high percentage of C_{20} and C_{22} PUFA, and the composition of these fatty acids was altered to a significant extent by n-3 dietary fatty acid modification (Fig. 1B) ($P < 0.05$). As a percentage of total fatty acids in PE, arachidonic acid was 30 and 6% in the safflower oil- and fish oil-fed groups, respectively, while 20:5n-3 and 22:6n-3 in these same groups were undetectable to 6% and 1.8 to 30%, respectively. The major difference between linseed oil and fish oil feeding was not in the relative quantity of 20:5n-3, but in the ratio of 22:5n-3 to 22:6n-3, which was approximately 1.5 in linseed oil-fed mice and 0.5 in fish oil-fed mice.

The major portion of the fatty acids in PS was 18:0, which represented approximately 50% of the total fatty acids. The relative compositional changes in PS PUFA were similar to those in PE, but PS in lungs from all dietary groups was uniquely enriched in 20:3n-6 (Fig. 1C). In fact, 20:3n-6 was the fatty acid least affected in each of the phospholipid classes examined.

The proportion of arachidonic acid in PE and PI was higher than in PC or PS (Fig. 1), and the dietary fats affected arachidonic acid content in PE to the greatest extent.

Lipoxygenase metabolites produced by homogenized lungs. The major metabolites formed by lung homogenates from all dietary groups were those of 12-lipoxygenase, 12-HETE and 12-HEPE derived from arachidonic acid and eicosapentaenoic acid, respectively. There was approximately 80 and 60% less 12-HETE produced by lung homogenates from linseed oil- and fish oil-fed mice, respectively, than by those from safflower oil-fed mice. The production of 12-HEPE was greater in the lung homogenates from linseed oil-fed (33-fold greater) and fish oil-fed (50-fold greater) groups than in those of the olive oil-fed group. Total 12-lipoxygenase products (12-HETE plus 12-HEPE) were lowest in the olive oil-fed group. The production of lipoxygenase metabolites as a function of dietary fats (Fig. 2) paralleled the fatty acid composition of the lung phospholipids (Fig. 3), with the association being most closely reflected by the levels of arachidonic acid and eicosapentaenoic acid in PC.

The variation in synthesis of 15-lipoxygenase products in response to dietary fat modification was different from that of 12-lipoxygenase. The 15-lipoxygenase product from arachidonic acid, 15-HETE, was relatively low in lungs of olive oil-, linseed oil-, and fish oil-fed mice, being about 15% of that produced in lungs of safflower oil-fed mice (Fig. 2), and the 15-lipoxygenase product from eicosapentaenoic acid (15-HEPE) was not detected under our experimental conditions.

Composition of fatty acids in homogenized lung tissues. Unesterified PUFA were measured in freshly homogenized

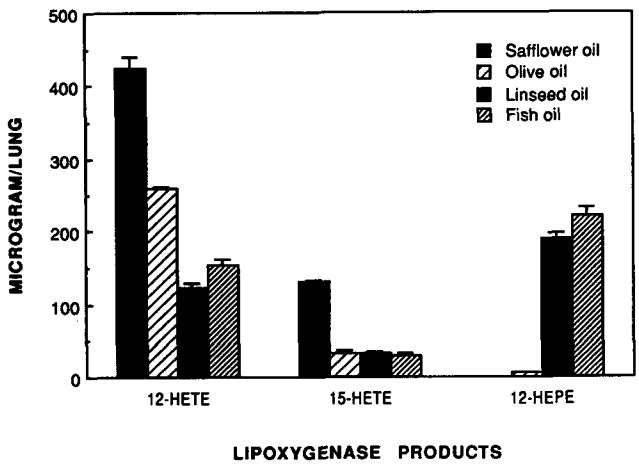


FIG. 2. Lipoxygenase products formed in incubated homogenates of lungs from mice fed diets varying in fat composition. Lipoxygenase products were extracted into ethyl acetate and methylated with diazomethane. After solid-phase extraction, the products were identified and quantified by reverse-phase high-performance liquid chromatography (n = 4). Bars are SD. 12-HETE, 12-hydroxyeicosatetraenoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid.

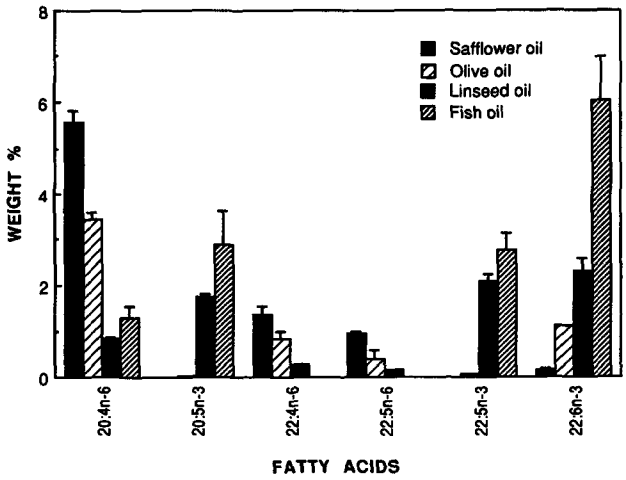


FIG. 3. Unesterified long-chain polyunsaturated fatty acids in homogenized lung tissue from mice fed diets varying in fat composition. Values are expressed as mol% of total free fatty acids (n = 4). Bars are SD.

lung tissue in the presence of the lipoxygenase inhibitor esculetin at 10 μM. Respectively, there was approximately 85 and 76% less arachidonic acid in lungs of linseed oil- and fish oil-fed mice than in lungs of safflower oil-fed mice, and 75- and 140-fold more eicosapentaenoic acid than in lungs of olive oil-fed mice (Fig. 3). Differences in unesterified arachidonic acid and eicosapentaenoic acid in fresh homogenized lung as a function of dietary fat source (Fig. 3) closely resembled their concentrations in isolated PC (Fig. 1A) and 12-lipoxygenase products in incubated homogenized lungs (Fig. 2).

Comparison of effects of dietary C_{18} and C_{20-22} n-3 PUFA. The shorter-chain and longer-chain dietary n-3 PUFA in linseed oil and fish oil, respectively, had similar effects on the modification of arachidonic acid content in phospholipids (Fig. 1). For mice fed linseed oil, which is rich in 18:3n-3, there was less arachidonic acid in PC (Fig. 1A), less unesterified arachidonic acid in fresh lung homogenates (Fig. 3), and less 12-HETE produced in incubated homogenized lung (Fig. 2) than for mice fed fish oil, which is rich in 20:5n-3 + 22:6n-3.

DISCUSSION

Experimental design. Previously, modification of tissue fatty acid composition and cell function in response to changes in dietary fatty acids was shown to be dependent on the quantity of other PUFA and total fat in the diet (21). Therefore, in the present study, the diets were varied solely in the type of unsaturated fatty acids and total fat was kept constant. As a consequence, total fat, total essential fatty acids and n-6/n-3 ratios were fixed relative to experimental conditions, and differences among dietary treatments solely reflected differences in the uptake and metabolism of the specific fatty acids.

Incorporation of n-3 PUFA and lung phospholipid composition. The fatty acyl composition of mammalian phospholipid membranes varies with tissue, cell type, and the composition of dietary fats (30). In lungs, dipalmitoyl PC is a major component of surfactant (31). Palmitic acid (16:0) represented approximately 80% of total fatty acids in the PC fraction of lungs from all animals used in this study, and there were no major differences among the dietary groups. This presumably reflects the low sensitivity of this phospholipid pool to modifications by dietary fatty acids. Thus, in spite of highly varied intakes of long-chain PUFA during growth and development, the accumulation of 16:0 in lungs was not affected.

The uptake of fatty acids into separate lipid classes and their subsequent metabolism are apparently regulated through a variety of pathways. Short-term modification of the diets of growing animals with structurally distinct fats enriched either in n-3, n-6, or n-9 PUFA exerts a significant effect on the composition of the membranes of animals (30). Dietary modification of membrane fatty acid composition has led to considerable interest in the possibility of modifying human diets for therapeutic or nutritional benefits. From this perspective, certain questions need to be addressed. Primarily, for therapeutic modulation, which fats should be included in diets? For example, is fish oil itself a uniquely effective component or do n-3 PUFA from other fat sources act equivalently? Previous studies in rodents (21) and humans (32) showed that diets rich in 18:3-n3 were less effective in modifying tissue concentrations of the longer-chain n-3 and n-6 PUFA than diets rich in 20:5n-3 and 22:6n-3, i.e., fish oils. This is clearly not due to an inability to metabolize 18:3n-3 into longer-chain products since the desaturases and elongases required are present in all animals examined (33). Therefore, it is possible that the differences in ability of shorter- (C_{18}) and longer- (C_{20-22}) chain n-3 PUFA to modify tissue PUFA concentrations are attributable to differences in the competition between n-3 and n-6 PUFA for metabolism to longer-chain products. That is, although 18:3n-3 can be metabolized efficiently to 20:5n-3 and 22:6n-3, this may not be realized if fluxes through the pathways are constant and the enzymes are simultaneously converting high levels of 18:2n-6. If true, such differences between shorter- and longer-chain n-3 PUFA potentially would be reversed if the shorter-chain n-3 PUFA were fed at higher levels and/or fed for longer time periods. The present study clearly demonstrated that the levels of arachidonic acid in lung phospholipids were responsive to n-3 dietary PUFA modification. At high and comparable n-3/n-6 ratios, linseed oil (rich in 18:3n-3) and fish oil (rich in 20:5n-3 and 22:6n-3) produced comparable effects on the modification of arachidonic acid contents in murine lung phospholipids. The higher 22:5n-3/22:6n-3 ratios observed in linseed oil-fed mice as compared to fish oil-fed mice suggest that the metabolism of 18:3n-3 by elongases and desaturases leads to the accumulation of longer-chain n-3 PUFA; however, the conversion of 22:5n-3 to 22:6n-3 by the pathway by which a Δ4 double bonds are introduced (34) seems to be limiting. In terms of lowering arachidonic acid levels during the developmental period of the animals, this suggests that shorter-chain and longer-chain PUFA have equivalent effects on murine lung phospholipids.

Arachidonic acid metabolism by 12-lipoxygenase. The potential role of 12-lipoxygenase metabolites in tumor cell adhesion and the possibility that enhanced arachidonic acid metabolism may account for a link between dietary n-6 PUFA metabolism and tumor metastasis led to this investigation of the relationships among dietary fat composition, lung lipid composition, and lipoxygenase products in lung tissue. The study confirmed that the majority of lipoxygenase products in lung were those of 12-lipoxygenase, i.e., 12-HETE from arachidonic acid and 12-HEPE from eicosapentaenoic acid (7–9). In addition, the data indicated that the formation of 12-lipoxygenase products was greatly affected by dietary n-3 PUFA modification. The shorter-chain n-3 PUFA (linseed oil) appeared to be more effective than longer-chain PUFA (fish oil) in decreasing 12-HETE production in incubated homogenized mouse lungs. This suggests that the production of 12-lipoxygenase products could be regulated by modification of fatty acyl composition of membrane phospholipids as a consequence of n-3 dietary PUFA manipulation. Although when 12-HETE was decreased by this means, a comparable increment in 12-HEPE was found. With respect to the involvement of 12-HETE in tumor cell adhesion, this could mean that manipulation of dietary fat is a nutritional approach to reduce the generation of this specific lipoxygenase metabolite *in vivo*; a net biological effect would depend upon differences in activity between 12-HETE and 12-HEPE.

Substrate pool(s) for lung 12-lipoxygenase. The complexity of oxygenase product formation *in vivo* can be attributed to various processes, including activation of specific phospholipases, release of arachidonic acid from discrete membrane phospholipids and conversion of arachidonic acid to metabolites by various oxygenases. Although release of arachidonic acid by phospholipases is believed to be the pivotal step in eicosanoid synthesis in many circumstances, it is not clear which phospholipids comprise the arachidonic acid pool in specific cell types of lung tissue. Distinct arachidonic acid pools may be used by different tissues or cells in response to particular stimulation *in vivo*, e.g., PE may be used in rat platelets (35) and PC and/or PI in mast cells (36). The present study compared fatty acid composition of lung phospholipids, lipoxygenase products in lung homogenates after 30 min of incubation, and composition of unesterified fatty acids in unincubated lung homogenates. These parameters were altered by modification of dietary PUFA. By comparing the extent in changes as a function of dietary fat, the data suggest that PC may serve as the major arachidonic acid pool for the 12-lipoxygenase pathway in mouse lung homogenates. As lung also expresses 15-lipoxygenase activity and as the response to diet for the 15-lipoxygenase in the mice differed, these two lipoxygenases may be controlled by different mechanisms. The physiological functions and differences in regulation mechanisms of mammalian lung lipoxygenases merit further investigation.

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Separation and Quantitation of Mono-, Di-, and Triglycerides and Free Oleic Acid Using Thin-Layer Chromatography with Flame-Ionization Detection[1]

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ABSTRACT: α-Monoolein was prepared from glycidol and oleic acid by the regioselective opening of glycidol in the presence of an anionic resin. During the reaction, the lipochemical synthesis medium becomes enriched in monoolein, the effective emulsifying agent. This mixture can be analyzed by thin-layer chromatography coupled with flame-ionization detection (FID). The products and reagents do not need to be derivatized. Diglyceride and triglyceride by-products affecting the selectivity of the reaction also could be detected using this technique. Cholesterol was used as an internal standard. The factors influencing the separation, including the hydrogen flow rate, scan speed, and the composition of the developing solvent, were investigated. The degree of separation is highly sensitive to the hexane/diethyl ether ratio of the developing solvent. Good separation of triglyceride, oleic acid, the two diglycerides, cholesterol, α-monoolein, and glycidol was obtained with the mixture hexane/diethyl ether/formic acid (65:35:0.04, by vol). Detector response, detection limits, and rod-to-rod variations also were examined. A range of rod loads giving a straightforward relationship between FID response and amount of compound loaded (relative to oleic acid and α-monoolein) was defined. The accuracy of the quantification was illustrated by analysis of a mixture of oleic acid and α-monoolein standards of known composition.

Lipids 31, 27–32 (1996).

Monoglycerides are used as additives for foods, feeds, cosmetics, coatings, and plastics (1–3). Stable oil-in-water or water-in-oil emulsions are produced under the action of those emulsifying agents. The normal reactions used to synthesize monoglycerides give rise to mixtures of mono-, di-, and triglycerides along with residual free fatty acids (1,4). The applications of these monoglycerides depend markedly on their overall content in the final product. The most commonly used methods for analysis of glycerides are gas-phase chromatography, thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC).

Prevot and Castille (5) analyzed monoglycerides by gas-phase chromatography on capillary columns. Although reproducible, this method requires derivatization of the hydroxyl groups on monoglycerides and fatty acids, and is also limited by the relatively short life-time of the column. Newer methods, including high-performance TLC (6) and high-performance size-exclusion chromatography (7), have been increasingly employed to separate and quantify partial glycerides in complex lipid mixtures.

TLC has the advantage of simplicity and flexibility in choice of operating parameters, although quantitation still presents problems. Amounts of products can be determined by densitometry, and, since the 1970s, coupling TLC with flame ionization has enabled single-step separation, identification, and quantification of each constituent of a complex lipid mixture.

Several workers have quantified the products of lipochemical reactions using TLC/flame-ionization detection (FID) (8,9). The partial glycerides from enzymatic hydrolysis of triolein (TO) have been determined by Tatara *et al.* (10) using boric acid-impregnated S-II chromarods (Iatron Laboratories, Tokyo, Japan) with a mobile phase of hexane/diethyl ether/acetic acid (70:30:1, by vol).

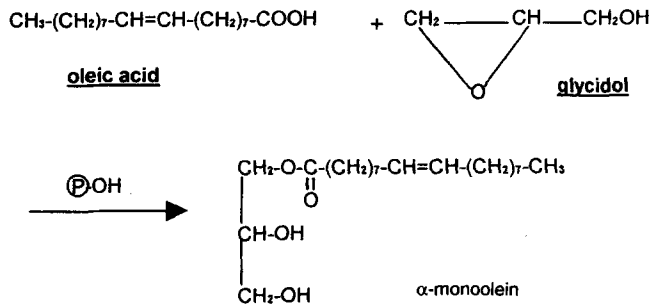
We propose a convenient method without requiring chemical derivatization of reagents or products with labile hydrogens, and without pretreatment of rods. It was developed using S-III (Iatron Laboratories) type rods. The mixtures to be analyzed were obtained by the condensation reaction of oleic acid with glycidol (GL) catalyzed by the anionic resin P-OH^- (Scheme 1) (11,12) in the absence of solvent. The α-monoolein (MO) was the main product formed in this fatty acid concentrated medium. By-products, such as diglycerides and triglycerides, formed in the reaction also were detected by this method. The TLC/FID analytical procedure was optimized using standard mixtures in order to assess the factors influencing the separation.

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Abbreviations: CHO, cholesterol; CV, coefficient of variation; 1,2-DO, 1,2-diolein; 1,3-DO, 1,3-diolein; GL, glycidol; HPLC, high-performance liquid chromatography; MO, α-monoolein; 18:1, oleic acid; TLC/FID, thin-layer chromatography/flame-ionization detection; TO, triolein.

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SCHEME 1

EXPERIMENTAL PROCEDURES

Reagents. Oleic acid (18:1) (>99%) was purchased from Fluka (L'Isle d'Abeau Chesnes, France). MO, TO, and cholesterol (CHO) of >99% purity were obtained from Sigma (L'Isle d'Abeau Chesnes, France). GL (>96%) was supplied by Aldrich (L'Isle d'Abeau Chesnes, France), and the anionic resin [Amberlyst A26 (OH^-)] was obtained from Rohm and Haas (Lauterbourg, France).

The lipochemical synthesis media resulted from the condensation of 18:1 with GL in a molar ratio of 18:1/GL (4:1, by mole). The reaction was carried out in the absence of solvent and was catalyzed by anionic resins. The reaction medium was thus referred to as a fatty acid concentrated medium. The amount of resin should correspond to a ratio 18:1/millliequivalent OH of the resin (50:5, by millimole). Initially, the resin was introduced into a flask fitted with stirrer and cooling system; 18:1 was then added to the resin and left for 15 min to adsorb at room temperature. GL was then added, and the reaction was continued for four hours at a temperature of 70°C. This produced 40% of MO in the final products. 1,2-Diolein (1,2-DO) and 1,3-diolein (1,3-DO) were obtained as by-products of the reaction. They were isolated from the synthesis media by column chromatography on Florisil (SDS, Peypin, France) with a hexane/diethyl ether mixture (50:50, vol/vol) as eluent (13).

Developing solvents. The solvent mixtures used for development contained X mL of hexane and 100-X mL of diethyl ether. Formic acid (0.04 to 1 mL) was added to 100 mL of the hexane/ether mixture. All solvents were HPLC grade.

Experimental procedure. Standards (TO, 18:1, 1,2-DO, 1,3-DO, CHO, MO, and GL) were dissolved as mixtures in HPLC-grade chloroform to obtain the desired concentrations. The concentration of the internal standard was fixed at 0.95 mg/mL for the assays.

A 1 mL of solution was spotted in fractions of 0.02 mL onto S-III chromarods with a model 3200/IS-01 autospotter (SES 3202; SES, Nieder-Olm, Germany). The S-III chromarods are reusable quartz rods coated with silica gel particles of uniform shape and size (14). Before spotting, the chromarods were scanned twice in the IATROSCAN (Iatron Laboratories, Tokyo, Japan; French distributor: BIONIS, Richebourg, France). Two sets of ten chromarods were simultaneously used in the experiments. After spotting, the set of chromarods was placed in the developing tank for 20 min. The rods were then dried in an oven at 120°C for 5 min, and then left in a vacuum dessicator for 5 min to reach room temperature. They were then transferred to the IATROSCAN and scanned.

Instrument and operating conditions. FID scans were performed using an IATROSCAN MK 5 analyzer connected to a 386 SX computer *via* an analog output. Data acquisition and analysis were carried out using Boreal software (JMBS Developments, Fontanil Cornillon, France).

The hydrogen and air flow rates of the FID were 160 and 1800 mL/min, respectively. The scanning speed was set at 3 corresponding to 35 s/rod. Other settings of hydrogen flow rate and scanning speed were only used in the experiments designed to study the influence of these two parameters.

RESULTS AND DISCUSSION

Influence of hydrogen flow rate. The response of the FID depends on the temperature of the flame, which is related to the hydrogen flow rate. Freedman *et al.* (15), working with S-II chromarods, chose a flow rate of 160 mL/min, which has been widely adopted by other authors (16–19). We experimented here with six different hydrogen flow rates over the range of 150–200 mL/min. The responses of 18:1 and MO at the different flow rates are shown in Figure 1. For these two compounds, hydrogen flow rates in the range of 160 to 180 mL/min gave the maximum response. However, high-flame temperatures may damage the silica coating of the rods, and so we selected a flow rate of 160 mL/min for the subsequent experiments.

Effect of scan speed. It has been reported that the response of some components increases with scan speed (20). However, complete burning may not be obtained at high scan speeds. Our normal scan speed was set at 3 corresponding to 35 s/rod. Two other scan speeds around this value were tested. As shown in Figure 2, only small alterations in the 18:1 and MO areas were observed between scan speeds 2 and 3, although a reduction was observed when the scan speed was

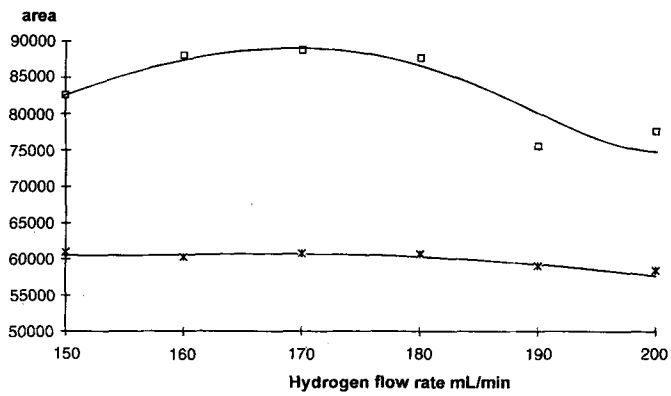


FIG. 1. Effect of hydrogen flow rate on response of oleic acid (18:1) (✳) and α-monoolein (MO) (□) spotted on S-III chromarods. 18:1 = 5 mg/mL, MO = 5 mg/mL; each point was the mean of values from ten rods.

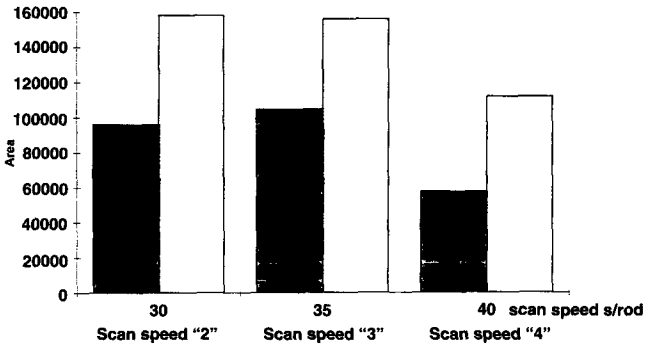


FIG. 2. Effect of scan speed on response of 18:1 (black bars) and MO (white bars). 18:1 = 5 mg/mL, MO = 5 mg/mL; each point was the mean of values from ten rods. Abbreviations as in Figure 1.

changed to 4. After spotting 5 mg of each component, no unburned sample was detected at any of these three scan speeds. A scan speed of 3 was thus selected for the subsequent experiments.

Effect of composition of developing solvent. The ternary mixture hexane/diethyl ether/formic acid is commonly used to analyze neutral lipid samples, containing fatty acids, tri-, di-, and monoglycerides (15), and good separations have been obtained using boric acid-impregnated S-II chromarods (10,21). However, the conditions described for the S-II rods are not directly applicable to the S-III chromarods, and pretreatment of the rods is not always required. Here, the optimum elution strength of the developing solvent mixture was optimized by adjusting the proportions of diethyl ether and formic acid. The effect of the hexane/diethyl ether ratio is shown in Figure 3.

As seen in Figure 3, the components can be considered in pairs—TO and 18:1, 1,2-DO and 1,3-DO, MO and GL. At low proportions of diethyl ether, TO and 18:1 were well resolved, but the 18:1 peak was broad. The two diglycerides were partially resolved, but MO and GL were not separated. Increasing the proportion of diethyl ether reduced the retention time of TO, 18:1, 1,2-DO, 1,3-DO, and MO without significantly influencing the retention time of GL. This enabled separation of MO and GL and improved the resolution of 1,2-DO and 1,3-DO without affecting the separation of TO and 18:1.

In another experiment, the hexane/diethyl ether ratio was kept constant, and different amounts of formic acid were added. The effect of formic acid is shown in Figure 4. Increasing the formic acid content enhanced the separation of MO and GL without affecting the separation of the other components. Peaks were sharper for 18:1, and especially for MO. The whole sample was well separated using hexane/diethyl ether (75:25, vol/vol) with various amounts of formic acid as developing solvent (Fig. 5A). However, with the internal standard (CHO), the 1,2-DO peak overlapped that of CHO (Fig. 5B).

More polar developing solvents were employed in an attempt to improve the separation of 1,2-DO and CHO. It was achieved with hexane/diethyl ether/formic acid (65:35:0.04, by vol) (Fig. 6). The diethyl ether content of the developing solvent may need to be altered slightly (±5% maximum) after several runs on the same rods if the quality of separation falls. This needs to be checked, especially when using new rods.

A complete separation of a mixture of TO, 18:1, 1,3-DO, 1,2-DO, MO, GL, and CHO was obtained in a single step without any chemical pretreatment of the chromarods, such as boric acid-impregnation. The S-III chromarods were found to give as good a separation as boric acid-impregnated S-II chromarods (10).

Calibration. The FID responses of 18:1 and MO were examined over a range of 0.08–20 μg of sample spotted, with hexane/diethyl ether/formic acid (75:25:0.04, by vol) as developing solvent. The detection limit was 0.01 μg for 18:1 and MO. In agreement with other investigators (20), we did not observe a linear relationship between the peak area and the amount of component spotted (Fig. 7). The FID response

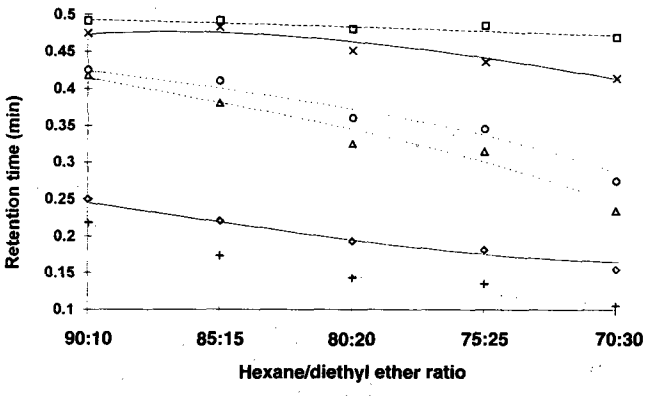


FIG. 3. Effect of hexane/diethyl ether ratio on the retention time of each component of the sample. Formic acid kept constant at 0.04 mL. Sample: triolein (TO) = 3 mg/mL, 18:1 = 3.2 mg/mL, 1,2-diolein (1,2-DO) and 1,3-diolein (1,3-DO) = 2 mg/mL, MO = 1.5 mg/mL, glycidol (GL) = 20 mg/mL (low response); 1 mL spotted; each point was the mean of values from six chromarods. Abbreviations as in Figure 1. TO, +; 18:1, ◇; 1,3-DO, △; 1,2-DO, ○; MO, ×; GL, □.

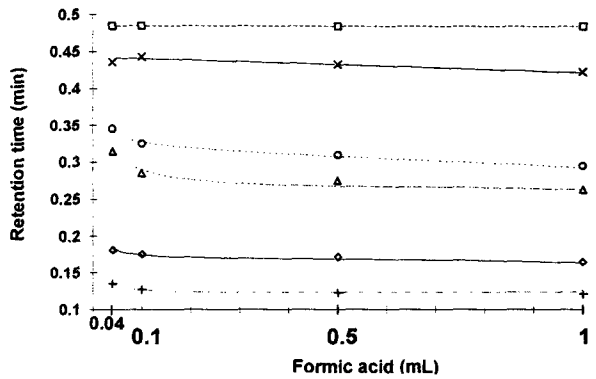


FIG. 4. Effect of formic acid on the retention time of each component of the sample hexane/diethyl ether ratio fixed at 75:25. Sample: TO = 3 mg/mL, 18:1 = 3.2 mg/mL, 1,2-DO and 1,3-DO = 2 mg/mL, MO = 1.5 mg/mL, GL = 20 mg/mL (low response); 1 mL spotted; each point was the mean of values from six chromarods. Abbreviation as in Figures 1 and 3, and key as in Figure 3.

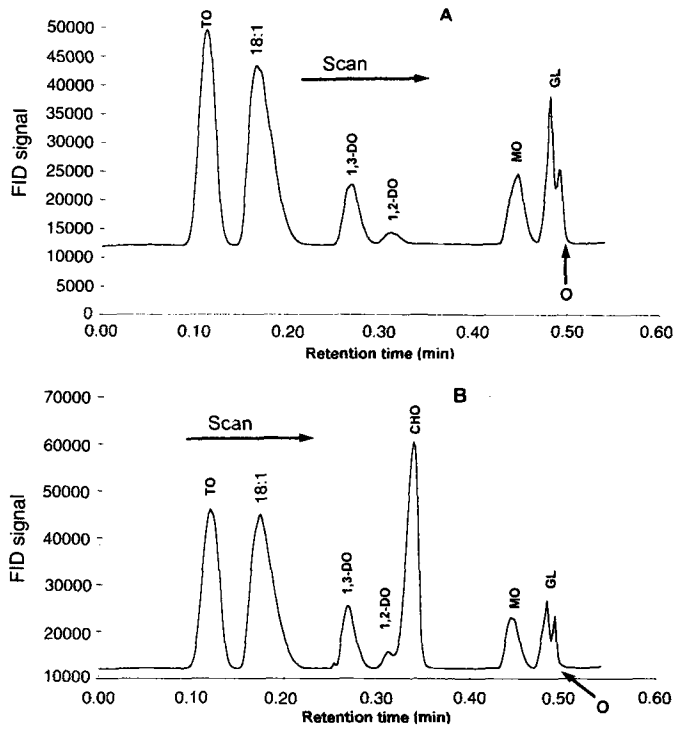


FIG. 5. Chromatograms of the sample developed with hexane/diethyl ether/formic acid (75:25:0.04). A: Without cholesterol (CHO); B: with CHO. Sample: TO = 3.2 mg/mL, 18:1 = 4.4 mg/mL, 1,2-DO and 1,3-DO = 1.5 mg/mL, MO = 1.5 mg/mL, GL = 18.6 mg/mL, CHO = 1 mg/mL; **O**: origin of sample application; GL produces a noisy signal close to the origin of sample application. Abbreviations as in Figures 1 and 3. FID, flame-ionization detection.

is in fact best fitted by power law equations: $Y = aX^b$ (20,22,23). The calibration curves of 18:1 and MO were approximated by the following equations: $Y = 8018X^{1.2204}$ (r^2 = 0.990) and $Y = 47819X^{0.5642}$ (r^2 = 0.880) for 18:1 and MO, respectively. A good correlation was found for 18:1 (r^2 = 0.990), but not for MO (r^2 = 0.880). The poor fit obtained for MO can be explained by a shift in the data over the range of 0.1 to 1 µg spotted (see zoom in Fig. 7). The data over the

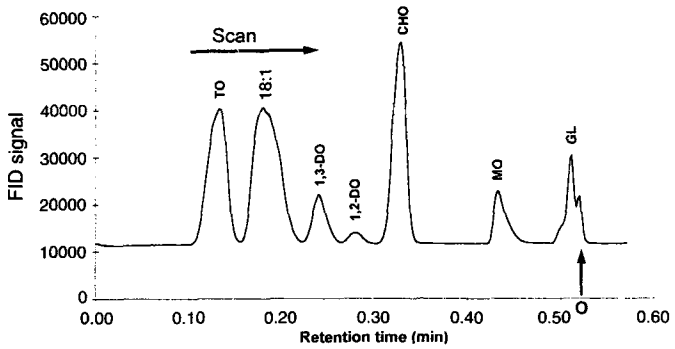


FIG. 6. Chromatograms of the sample developed with hexane/diethyl ether/formic acid (65:35:0.04). Sample: TO = 3.2 mg/mL, 18:1 = 4.4 mg/mL, 1,2-DO and 1,3-DO = 1.5 mg/mL, MO = 1.5 mg/mL, GL = 18.6 mg/mL, CHO = 1 mg/mL. Abbreviations as in Figures 1, 3, and 5.

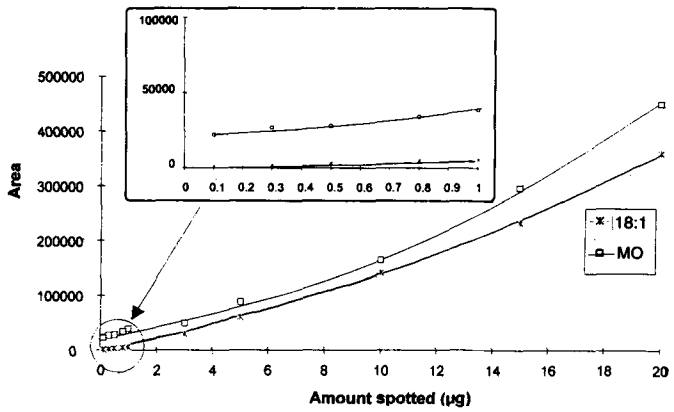


FIG. 7. Relationship between the peak area and the amount spotted for 18:1 and MO; 1 µL spotted; each point was the mean of values from ten rods. Abbreviation as in Figure 1.

range of 3–20 µg were well described by the equation $Y = 13626X^{1.1415}$ with a high correlation coefficient (r^2 = 0.992).

Fluctuations tended to occur and day-to-day reproducibility was not particularly good. This was accounted for by changes in room humidity. When the chromarods are exposed to the atmosphere, the silica gel coating quickly adsorbs moisture (24) and the percentage of water adsorbed influences the activity of the rod.

Sebedio and Juaneda (25) found that good results could be obtained by multilevel calibrations using an internal standard. The power law regression can be replaced by a linear regression over a defined range of component loads without affecting the correlation coefficient. As shown in Figure 8, interrod

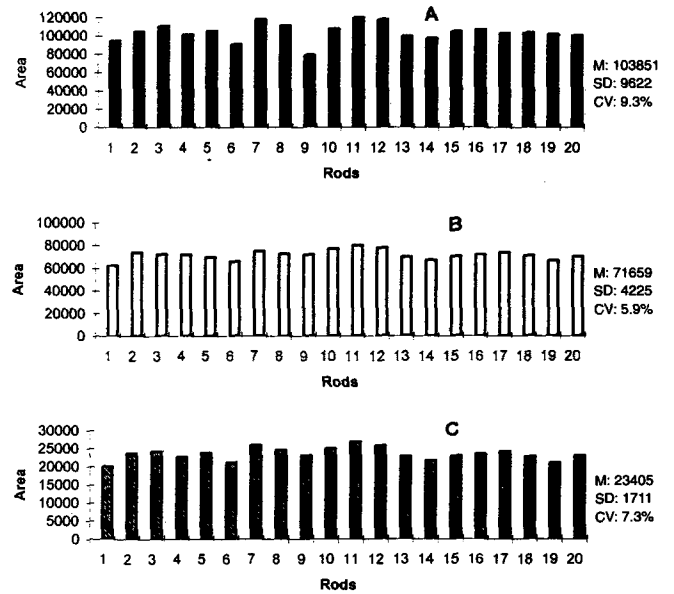


FIG. 8. Response of the two sets of ten chromarods. A: MO (4 µg spotted); B: 18:1 (4 µg spotted); C: CHO (0.95 µg spotted). M, mean of areas; SD, standard deviation; CV, coefficient of variation. Abbreviations as in Figures 1, 5, and 8.

variations were observed for 18:1 [coefficient of variation (CV) = 5.9%], MO (CV = 9.3%), and CHO (CV = 7.3%). Similar shapes were obtained for the three components 18:1, MO, and CHO over 20 chromarods. The use of an internal standard thus reduces interrod dispersion, and the responses of MO and 18:1 were normalized to the response of the internal standard (CHO) (Fig. 9), producing a significant reduction in the interrod variation. The CV was reduced from 9.3 to 6.3% for MO and from 5.9 to 2.5% for 18:1.

The relationships between the peak area and weight ratios were approximated by a linear regression over the range of 0.8 to 8 μg spotted for 18:1 and MO as governed by the following equations: $Y = 0.834X - 0.436$ with $r^2 = 0.996$ for 18:1 and $Y = 1.079X - 0.358$ with $r^2 = 0.995$ for MO. The high correlation coefficients indicated a good fit, and the plots are shown in Figure 10.

Analysis of a sample of known composition. A mixture of known composition of 18:1, MO, and CHO was prepared; 18:1 and MO were made up at concentrations of 4.6 mg/mL, and the internal standard at 0.95 mg/mL. Ten rods were used in the analysis of this model sample. The concentrations of 18:1 and MO were determined using the linear regressions established from the previous experiment, and the results are listed in Table 1.

The concentrations of 18:1 and MO were calculated from the means of the values from the ten rods. There was an adequate agreement between the observed results and the known concentrations of the components in the sample. However, the high CV observed for MO (10%) demonstrated that ten rods are required to achieve a relatively accurate determination. An analysis based on three rods would not be satisfactory.

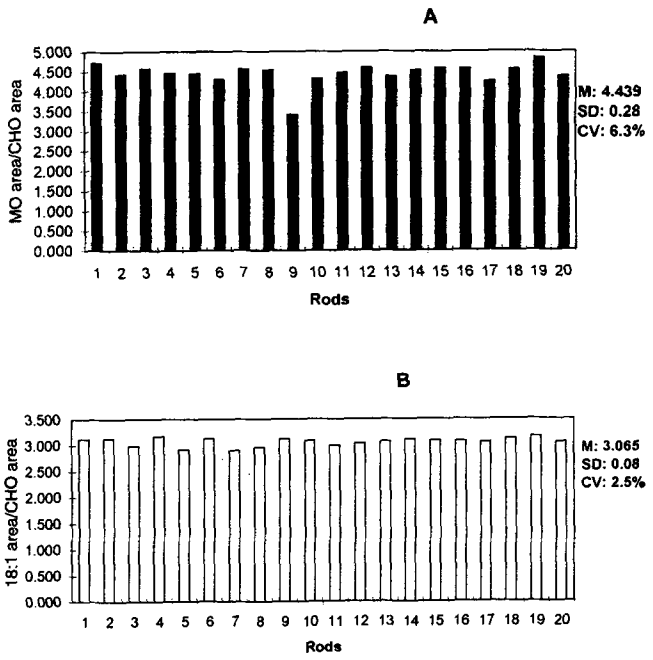


FIG. 9. Normalized response of the two sets of ten chromarods. A: MO/CHO; B: 18:1/CHO. Abbreviations as in Figures 1, 5, and 8.

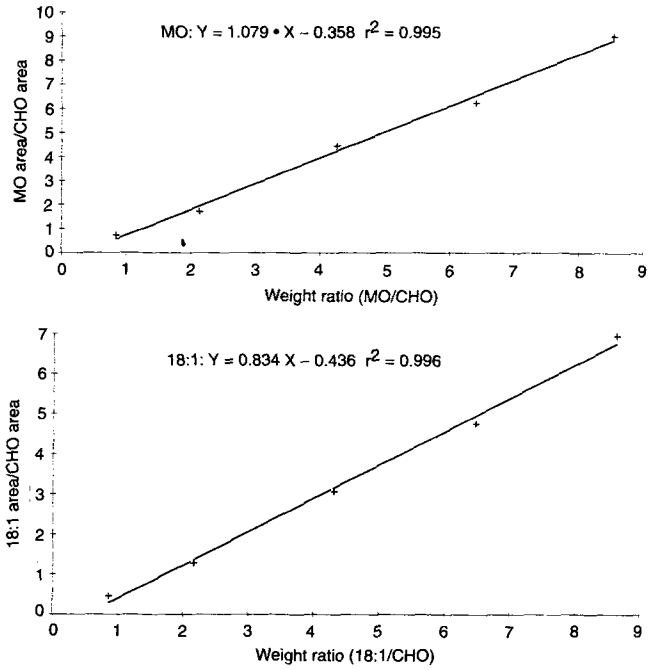


FIG. 10. The relationship between the peak area and weight ratios for MO and 18:1 with respect to the internal standard (CHO). Abbreviations as in Figures 1 and 5.

This analytical procedure using TLC/FID was found to be a rapid and efficient method for the analysis of the typical GL mixtures found in our synthesis media. Complete separation of the mixture was achieved in a single step without pretreatment of the chromarods and without derivatization of the sample. A hydrogen flow rate of 160 mL/min and a scan speed of 35 s/rod were found to be the optimum parameters for the IATROSCAN apparatus.

We obtained complete separation of the standards mixture (TO, 18:1, 1,2-DO, 1,3-DO, CHO, MO, and GL) using a developing solvent of hexane/diethyl ether/formic acid (65:35:0.04, by vol). However, for prolonged reuse of chromarods, the elution strength of the developing solvent may

TABLE 1
Analysis of a Model Mixture of 18:1 and MO[a]

Rod	18:1 (mg/mL)	MO (mg/mL)
1	4.49	5.57
2	4.52	4.42
3	4.46	4.88
4	4.60	5.17
5	4.19	5.06
6	4.53	4.33
7	4.20	4.77
8	4.49	4.15
9	4.23	5.34
10	4.22	4.59
Mean	4.4	4.8
SD	0.16	0.46
CV%	4	10

[a]$C_{18:1}$ = 4.6 mg/mL; α-monoolein (MO) = 4.6 mg/mL; cholesterol = 0.95 mg/mL. SD, standard deviation; CV, coefficient of variation (%).

need to be altered for optimum separation. An internal standard is needed for satisfactory reproducibility. Regular calibration with samples of known composition is also required.

The IATROSCAN MK-5 using S-III chromarods thus appears to be well suited for TLC/FID analysis of neutral lipids. It is noteworthy that this analytical technique requires relatively little maintenance, and several developments can be carried out using only 100 mL of solvent.

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Optimization of the Mass Spectrometric Analysis of Triacylglycerols Using Negative-Ion Chemical Ionization with Ammonia

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ABSTRACT: Conditions for the mass spectrometric analysis of triacylglycerols, *via* direct exposure probe, with ammonia negative-ion chemical ionization were optimized. Triacylglycerols were most favorably ionized, using the reactant gas pressure of approximately 8500 mtorr at the ion source temperature of 200°C with the instrumentation used. Abundant $[M - H]^-$ ions were produced under these conditions without the formation of $[M + 35]^-$ cluster ions, which would interfere with the molecular weight region of triacylglycerols in the spectra. A rapid desorption of the sample from the probe wire is recommended, using a relatively high heating rate (approximately 40 mA s^{-1}), to minimize thermal degradation of unsaturated molecules and the reducing effect of double bonds on the mass spectrometric response of triacylglycerols. Furthermore, the abundance of $[M - H]^-$ ion was enhanced by rapid heating, which we found to be important to improve the sensitivity. The appropriate amount of sample applied to the rhenium wire was in the region of 50–300 ng for one determination, i.e., only a few nanograms of a single triacylglycerol is required for production of a reliable spectrum. The reproducibility of the method was good as demonstrated with standards and a raspberry seed oil sample. The described mass spectrometric method is a fast and potentially useful tool for semiquantitative determination of triacylglycerol mixtures of various fats and oils. The discrimination, caused by differences in molecular size and unsaturation of triacylglycerols with 50 to 56 acyl carbons, was negligible under our optimized ionization conditions, thus, no correction factors were needed. These findings have not yet been verified with instruments in other laboratories. However, the present study shows how the analysis of triacylglycerols can be improved, regardless of the instrument, by optimization of the analytical conditions.

Lipids 31, 33–42 (1996).

The first mass spectrometric studies of triacylglycerols (TAG) in the 1960s were performed with electron ionization (EI) impact (1,2). In addition to fragment ions, the spectra consisted of $M^{+\bullet}$ and $[M - 18]^+$ ions at low abundance, which were used to determine the molecular weight distribution of TAG of several vegetable oils (3,4). As compared with EI spectra, a greater abundance of ions, consisting of the intact molecule, and less fragmentation are obtained by soft ionization methods, chemical ionization (CI) being used most often (5,6). TAG have been ionized using methane, isobutane, or ammonia as a reactant gas in the positive-ion mode to produce $[M + H]^+$ or adduct ions. Murata and Takahashi (7,8) ionized TAG with ammonia, which resulted in the formation of abundant $[M + NH_4]^+$ ions and $[M + H - RCO_2H]^+$ fragment ions useful for the determination of both molecular weights and combinations of fatty acids in TAG molecular species. Kuksis and colleagues (9,10) introduced the formation of prominent $[M + H]^+$ and $[M + H - RCO_2H]^+$ ions when TAG were separated by reversed-phase high-performance liquid chromatography (HPLC) combined with mass spectrometry (MS) using acetonitrile and propionitrile for elution and as reactant gases. The detection sensitivity was greatly improved by using negative-ion CI with dichloromethane, which yielded solely $[M + Cl]^-$ ions (11,12).

It has been shown recently by Kallio and Currie (13,14) that MS utilizing ammonia negative-ion CI is an efficient tool for the analysis of TAG. NH_2^- ion is a strong Brønsted base which is capable of deprotonating most organic molecules, including ketones and esters (15,16). TAG will be ionized to form deprotonated ions using ammonia as the reactant gas in negative ion mode. The *m/z* values of $[M - H]^-$ ions define unambiguously the number of acyl carbons and the number of double bonds present in the TAG of most fats and oils, and the abundance of $[M - H]^-$ ions determines the proportions of different molecular weight species. Similar data cannot be achieved with a single mode of either gas chromatography (GC) or HPLC, which are the most widely utilized techniques in lipid research. In addition, more detailed information on molecular species is obtained by collision-induced dissociation of a selected $[M - H]^-$ ion. The daughter-ion spectrum provides information on the fatty acid constituents and their distribution between *sn*-2 and *sn*-1,3-positions in TAG.

In this research, the conditions for mass spectrometric analysis of TAG of seed oils using ammonia negative-ion CI were optimized. Samples were introduced *via* a direct exposure probe into the ion source without chromatographic separation before MS. First of all, the effects of ion source temperature and reactant gas pressure, as well as the rate of probe heating on the quality of ammonia negative-ion CI mass spec-

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Abbreviations: CI, chemical ionization; EI, electron ionization; GC, gas chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; TAG, triacylglycerols.

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tra of TAG, were studied. In addition, the reproducibility of the method and its applicability for quantitative analysis of TAG were investigated.

EXPERIMENTAL PROCEDURES

TAG standards and samples. Standards of both saturated and unsaturated TAG, listed in Table 1, were purchased from Sigma (St. Louis, MO). A stock solution of each compound (approximately 0.2–0.4 mg mL^{-1}) was prepared by dissolving the saturated TAG in dichloromethane and the unsaturated ones in hexane containing 0.02% (wt/vol) butylated hydroxytoluene (Sigma) to prevent oxidation. Individual components or mixtures of several standards were dissolved in hexane for mass spectrometric determinations. Lipids in the seeds of black currant (*Ribes nigrum*), lingonberry (*Vaccinium vitis-idaea*), and raspberry (*Rubus idaeus*), grown wild in Finland, were extracted with chloroform/methanol (2:1, vol/vol) using a modified Folch procedure (17) followed by purification of the TAG by elution from a short column of Florisil™ (Fluka Chemie AG, Buchs, Switzerland) with 10 mL hexane/diethyl ether (4:1, vol/vol) as the mobile phase. TAG were dissolved in hexane (approximately 1 mg mL^{-1}) for MS determinations. All solvents used were HPLC-grade supplied by Rathburn (Walkerburn, Scotland) and Merck (Darmstadt, Germany).

Mass spectrometric analysis. The mass spectrometric determinations were conducted with a Finnigan MAT TSQ-700 triple quadrupole instrument (San Jose, CA) with an ICIS II data system (Finnigan MAT). TAG were analyzed using negative-ion CI with ammonia (≥99.998%; Prax Air, Oevel, Belgium). The optimization of the ion source conditions was carried out with a mixture of four TAG standards at ammonia pressures of 3000, 5000, 7000, 8500, and 10000 mtorr at ion source temperatures of 120, 150, 180, 200, and 220°C. The ammonia pressure was monitored with a Convectron gauge (Phillips Company, Granville, OH) which was situated in the gas line before the flow-controller into the ion source. A volume of 0.5 μL of the sample was applied to the rhenium wire loop of the direct exposure probe (Finnigan MAT). The probe was introduced into the ion source after the solvent was evaporated, and the heating of the probe wire with the current was started to vaporize the sample. The desorption of TAG from the rhenium wire was optimized by testing heating rates between 2.5 and 100 mA s^{-1} both with mixtures of TAG standards and seed oil samples. The effect of concentration on the mass spectrometric response of TAG was tested with a mixture of 14 standards (mixture A, Table 1) with concentrations varying from 2.6 to 379.6 μmol L^{-1} of each compound. The concentration of the internal standard, *rac*-18:0-18:0-14:0, was constant (24.7 μmol L^{-1}) in each dilution. The repeatability and reproducibility of the mass spectrometric method was determined with TAG standards (mixture B, Table 1) and raspberry seed oil. Electron energy was 70 eV and filament current 400 μA throughout the experiment. The *m/z* range scanned was 250–950 during the optimization of the ion source conditions and that of the probe heating rate, and 700–1100 during the concentration, repeatability, and reproducibility experiments. Scans containing ions representing deprotonated TAG were averaged in each analysis. The abundances of the ions were corrected by ^{13}C isotope content, when necessary, before expressing the results as the mean of four analyses.

RESULTS AND DISCUSSION

Ammonia negative-ion CI of TAG yielded very simple mass spectra containing abundant $[M - H]^-$ ions. The *m/z* values and abundances of $[M - H]^-$ ions define the number of acyl

TABLE 1
Reference Triacylglycerols and Their Abbreviations Used in This Study

Triacylglycerols	Abbreviation	ACN:*n*[a]	Mixture A (ng μL^{-1})[b]	Mixture B (ng μL^{-1})[b]
Saturated				
Tripalmitoylglycerol	16:0-16:0-16:0	48:0	210	420
rac-1,2-Distearoyl-3-myristoyl-*sn*-glycerol	*rac*-18:0-18:0-14:0	50:0	–[c]	412
Triheptadecanoylglycerol	17:0-17:0-17:0	51:0	212	424
rac-1,2-Distearoyl-3-palmitoyl-*sn*-glycerol	*rac*-18:0-18:0-16:0	52:0	202	404
Tristearoylglycerol	18:0-18:0-18:0	54:0	212	424
Trinonadecanoylglycerol	19:0-19:0-19:0	57:0	210	420
Trieicosanoylglycerol	20:0-20:0-20:0	60:0	206	412
Unsaturated				
rac-1-Palmitoyl-2-oleoyl-3-stearoyl-*sn*-glycerol	*rac*-16:0-18:1-18:0	52:1	200	200
rac-1,2-Dioleoyl-3-palmitoyl-*sn*-glycerol	*rac*-18:1-18:1-16:0	52:2	326	652
rac-1,2-Distearoyl-3-oleoyl-*sn*-glycerol	*rac*-18:0-18:0-18:1	54:1	194	388
rac-1,2-Dioleoyl-3-stearoyl-*sn*-glycerol	*rac*-18:1-18:1-18:0	54:2	287	1148
Trioleoylglycerol	18:1-18:1-18:1	54:3	212	424
rac-1,2-Dilinoleoyl-3-oleoyl-*sn*-glycerol	*rac*-18:2-18:2-18:1	54:5	206	412
Trilinoleoylglycerol	18:2-18:2-18:2	54:6	236	472
Trilinolenoylglycerol	18:3-18:3-18:3	54:9	188	752

[a]ACN is the combined number of acyl carbons in a triacylglycerol, *n* is the combined number of double bonds in the three acyl chains of a triacylglycerol.
[b]Composition of the stock solution.
[c]Internal standard (20.6 ng μL^{-1}) added to each dilution in concentration experiments.

carbons and double bonds in TAG and the proportions of different molecular weight species. During this optimization study, more detailed information on the effects of different parameters on the analysis of TAG was considered important in order to expand the applicability of the method. The samples were introduced *via* a direct exposure probe into the ion source. The evaporation of different TAG does not occur simultaneously from the rhenium wire (18); therefore, the ion abundances obtained from the averaged spectra were used for further calculations.

Optimization of the ion source conditions. It is well known that ion source conditions may have a significant influence on the composition of the reactive CI gas plasma and, consequently, on the resulting mass spectra of sample molecules (16). The aim of the optimization was to find ion source conditions which will yield maximum sensitivity of deprotonated ions of TAG at minimum ion source pressure without formation of interfering cluster ions. The test mixture consisted of *rac*-1,2-distearoyl-3-palmitoyl-*sn*-glycerol, tristearoylglycerol, *rac*-dilinoleoyl-3-oleoyl-*sn*-glycerol, and trilinolenoylglycerol, each compound in the concentration of 0.15–0.17 $\mu g\ \mu L^{-1}$ in hexane, representing typical components in the seed oils of various plants, especially in the seeds of Northern wild berries. TAG containing 52, 54, and 56 acyl carbons with 1–9 double bonds are the most abundant constituents of several seed oils.

Ammonia negative-ion CI of TAG resulted in a spectrum which contained $[M - H]^-$ and $[M + 35]^-$ ions at various proportions, depending on the CI conditions in the ion source. In addition, only abundant RCO_2^- ions were produced with little structural significance and, therefore, are not discussed here. Figure 1A shows the abundance of $[M - H]^-$ ions originated from trilinolenoylglycerol at different ion source conditions. The ionization of *rac*-1,2-distearoyl-3-palmitoyl-*sn*-glycerol, tristearoylglycerol, and *rac*-1,2-dilinoleoyl-3-oleoyl-*sn*-glycerol were similar to that presented in Figure 1A. In general, the ionization efficiency decreased with increasing ion source temperature at constant CI gas pressure. At constant ion source temperature, the ionization efficiency of TAG increased with increasing reactant gas pressure up to 8500 mtorr. Some day-to-day variation existed in the ionization of the TAG, e.g., sometimes the ionization efficiency at 8500 and 10000 mtorr was nearly the same.

The proportion of the abundance of the cluster ion $[M + 35]^-$ from that of the corresponding $[M - H]^-$ ion originated from trilinolenoylglycerol is shown in Figure 1B. The formation of cluster ions during the analyses of *rac*-1,2-distearoyl-3-palmitoyl-*sn*-glycerol, tristearoylglycerol, and *rac*-1,2-dilinoleoyl-3-oleoyl-*sn*-glycerol at different ion source conditions resembled that shown in Figure 1B. Typically, the contribution of cluster ions decreased with increasing reactant gas pressure and increasing ion source temperature. For example, the proportions of cluster ions were more than 10% of the corresponding $[M - H]^-$ ion at the pressure of 3000 and 5000 mtorr at the ion source temperature of 120°C for each TAG studied. On the other hand, no cluster ions were observed at temperatures of 200 and 220°C when the intensities of the ions of interest were derived from the averaged scans.

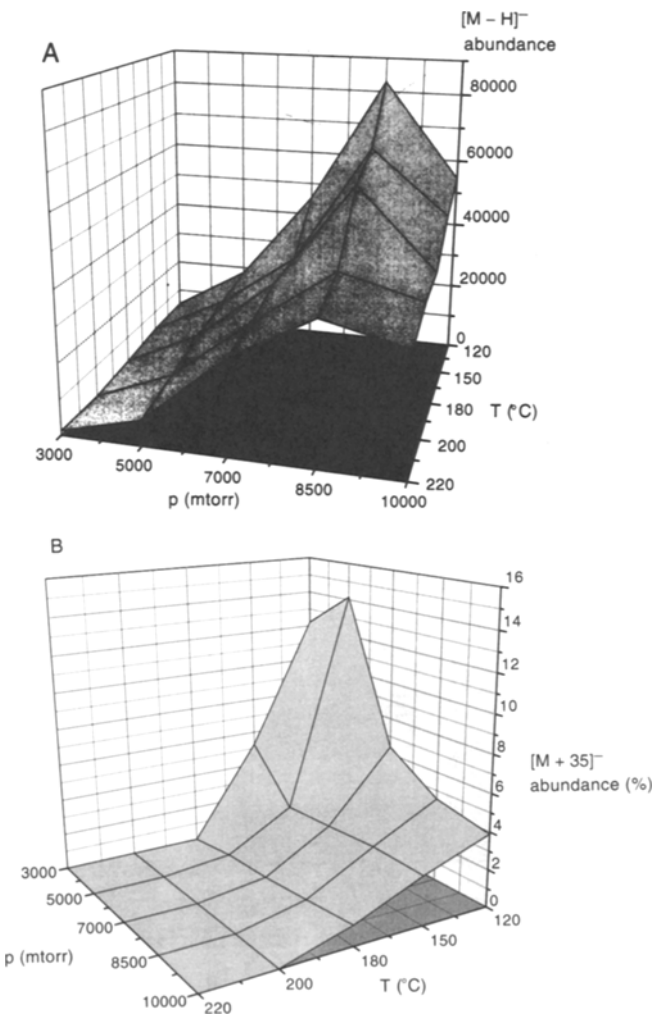


FIG. 1. A: The abundance of $[M - H]^-$ ion at different ion source conditions. B: The proportion of the abundance of the cluster ion, $[M + 35]^-$, from that of the corresponding deprotonated molecular ion at different ion source conditions. Sample: trilinolenoylglycerol; T, temperature; p, pressure.

Based on the ionization efficiency of TAG, the reactant gas pressure should be high and the ion source temperature low for maximal ionization. On the contrary, the formation of cluster ions is favored at low temperatures. Cluster ions disturb the molecular weight area of TAG in the spectra; thus, the formation of clusters should be eliminated with high enough ion source temperatures. TAG were most favorably ionized using the reactant gas pressure of approximately 8500 mtorr at the ion source temperature of 200°C with the instrument used, for the determination of molecular weight distribution of fats and oils. These conditions may serve as a guideline for optimization of the CI conditions for TAG, but, it may be necessary to make adjustments for these conditions depending on the sample and instrumentation. All experimental measurements described below were carried out at the optimized CI conditions.

Optimization of the probe heating rate. The effect of the heating rate of the rhenium wire of the direct exposure probe was studied with single standards (tristearoylglycerol, *rac*-1,2-dilinoleoyl-3-oleoyl-*sn*-glycerol, and trilinolenoylglycerol), a fifteen-component reference mixture (mixture B, diluted 1:9 with hexane, Table 1), black currant, and lingonberry seed oils. Unsaturated TAG are the most abundant constituents in most seed oils, containing even more than nine double bonds in their acyl chains. Therefore, it is of great significance to reduce thermal degradation or polymerization of these unsaturated molecular species during their desorption from the rhenium wire in the ion source. Heating rates from 2.5 to 100 mA s^{-1} were tested in this study. As an example, the influence of the probe heating rate on the proportions of RCO_2^-, $[M - RCO_2]^-$, and $[M - H]^-$ ions of the reference compound *rac*-1,2-dilinoleoyl-3-oleoyl-*sn*-glycerol is shown in Figure 2. We assumed that the proportions of RCO_2^- and $[M - RCO_2]^-$ ions would indicate the degree of thermal degradation of TAG. Surprisingly, the intensities of fragments resulting from the acyl loss of reference TAG, $[M - RCO_2]^-$, were very low, if found at all. The proportions of RCO_2^- and $[M - H]^-$ did not change significantly according to the rate of probe heating. However, the higher the heating rate the higher the abundance of $[M - H]^-$ ions. Such enhanced signal of the $[M - H]^-$ ions is essential to improve sensitivity. Very high heating rates resulted in bad peak shapes and high standard deviations because few data points only were acquired.

The effect of probe heating rate on the proportions of TAG of the fifteen-component reference mixture, according to the intensities of $[M - H]^-$ ions, is presented in Figure 3A. The proportions of unsaturated TAG with more than three double bonds were underestimated with a low heating rate of the probe. However, similar effect was not determined with the analyses of TAG of black currant seed oil (Fig. 3B) and lingonberry seed oil (Fig. 3C), although they contained around 62 and 82% TAG with six or more double bonds, respectively. An increase in the heating rate resulted in slightly reduced proportions of larger TAG, whereas those of smaller molecules increased. Overall, the heating rate of the probe did not significantly change the TAG distribution of the seed oils studied. It appears most appropriate to use a heating rate of around 40 mA s^{-1} of the direct exposure probe wire for the vaporization of TAG to obtain good sensitivity and reproducibility of the analysis and to minimize the discrimination of polyunsaturated TAG.

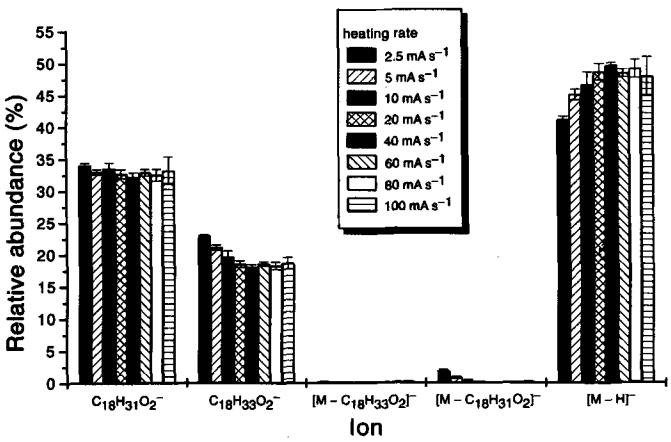


FIG. 2. The effect of the heating rate of the direct exposure probe on the proportions of RCO_2^-, $[M - RCO_2]^-$, and $[M - H]^-$ ions of *rac*-1,2-dilinoleoyl-3-oleoyl-*sn*-glycerol. Reactant gas pressure, 8500 mtorr; and ion source temperature, 200°C.

Effect of the sample amount. The effect of sample amount on the mass spectrometric response of TAG was tested with several dilutions of a mixture of reference components (mixture A, Table 1) concentrations varying from 2.6 to 379.6 μmol L^{-1} of each compound. A constant volume of a sample (0.5 μL) was applied to the rhenium wire; therefore, the amount of sample analyzed was changed by varying the concentration of the sample. An internal standard, *rac*-18:0-18:0-14:0, was added to each dilution (24.7 μmol L^{-1}) to eliminate inaccuracies in injection volumes.

The mass spectrometric response of the $[M - H]^-$ ions of TAG varied a lot as a function of the molecular size, with a slow probe heating (Fig. 4A). The response decreased with increasing molecular size, i.e., the total number of acyl carbons in TAG. As expected, the double bonds had a diminishing effect on the mass spectrometric response of unsaturated TAG (Fig. 4B). In addition to concentration, the effect of probe heating rate on the proportions of TAG was studied using slow (4 mA s^{-1}) and rapid (80 mA s^{-1}) heating (Fig. 5). Correction factors for each standard were calculated in relation to tripalmitoylglycerol (48:0) to determine the potential of this method for quantitative purposes (Table 2). When the sample was vaporized slowly, the proportions of unsaturated TAG were underestimated, being lower the more dilute the sample was. This resulted in large correction factors for unsaturated components (Table 2). The discrimination of polyunsaturated TAG can be eliminated by using rapid heating of a sample and a small sample amount. In this case, the correction factors of both saturated and unsaturated TAG were close to 1.0 (Table 2), i.e., the measured distribution of TAG was close to that of the composition of the mixture. The analysis of high-concentration samples using rapid heating of the probe resulted in increased proportions of smaller TAG and decreased proportions of larger molecules, as well as unsaturated components. Minor inaccuracies in the correction factors might be caused by sample preparation and impurities of the standards.

A few studies on the quantitative analysis of TAG with direct introduction inlet mass spectrometry have been published utilizing either EI (3) or ammonia CI (8,18). In these studies, the mass spectrometric response of TAG was reported to depend on the molecular structure, i.e., number of double bonds and molecular size; thus, correction factors had to be used for quantitative measurements. The results of the present study clearly show that the variation in the mass spectrometric response of TAG caused by differences in unsaturation and molecular size could be reduced, and nearly eliminated, by heating the probe wire very rapidly (Table 2), if the sample

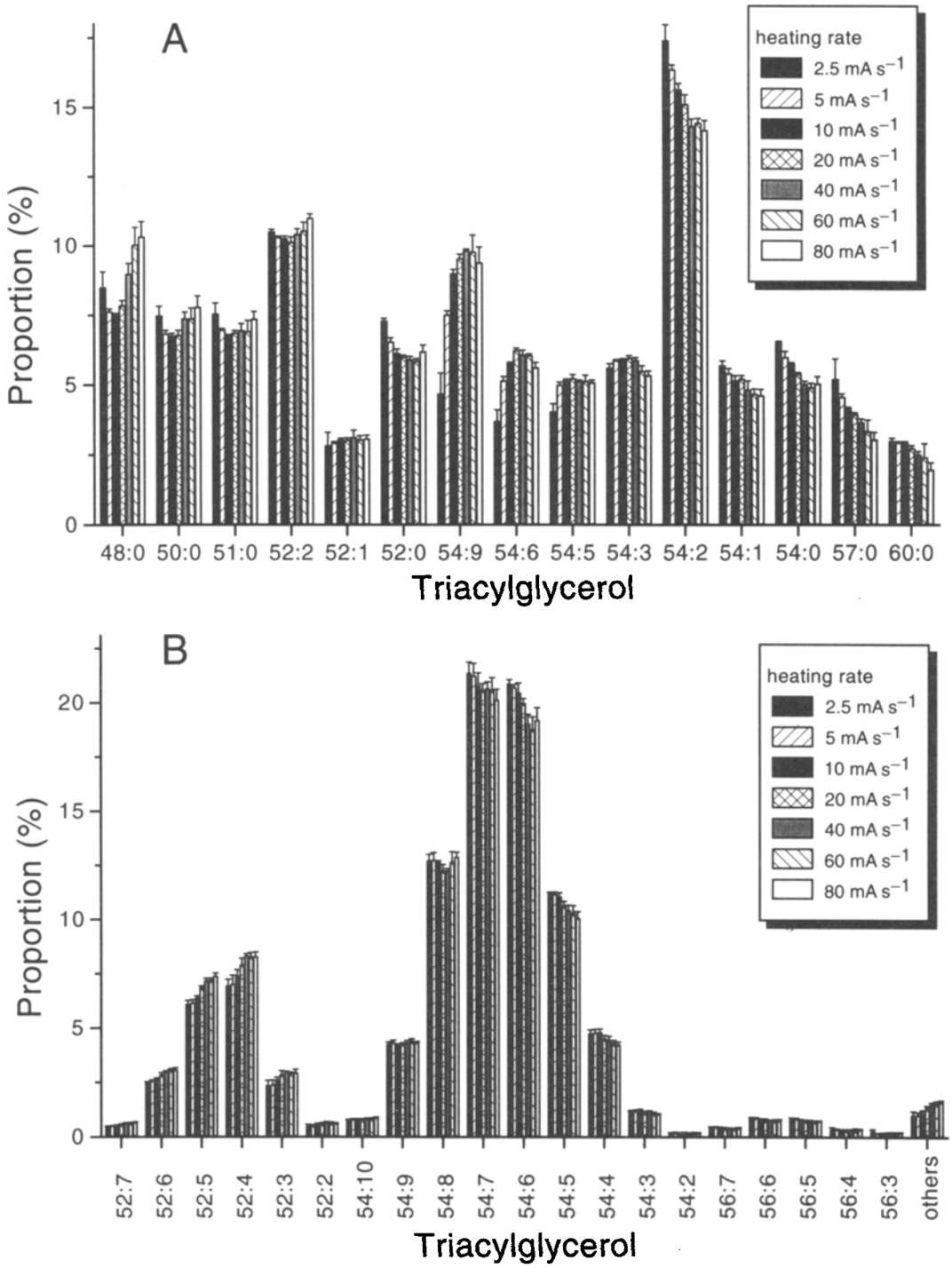


FIG. 3. The effect of the heating rate of the direct exposure probe on the distribution of triacylglycerols: A, reference mixture B (see Table 1), diluted 1:9 with hexane; B, black currant seed oil; and C, lingonberry seed oil. The proportions of triacylglycerols are based on the ^{13}C corrected abundances of $[M - H]^-$ ions of four analyses. Reactant gas pressure, 8500 mtorr; and ion source temperature, 200°C.

amount was small enough. Too small an amount of sample resulted in unacceptable standard deviation of the analysis. Based on the analysis of the TAG reference mixture, the appropriate concentration of the sample was approximately 100–600 ng μL^{-1}, i.e., 50–300 ng TAG per determination. In other words, only a few nanograms of a single TAG is required for production of a reliable spectrum. It is necessary to adjust the concentration of the sample to a range suitable for the analysis in order to avoid too large sample amounts. To compare TAG distributions, the concentration of each sample

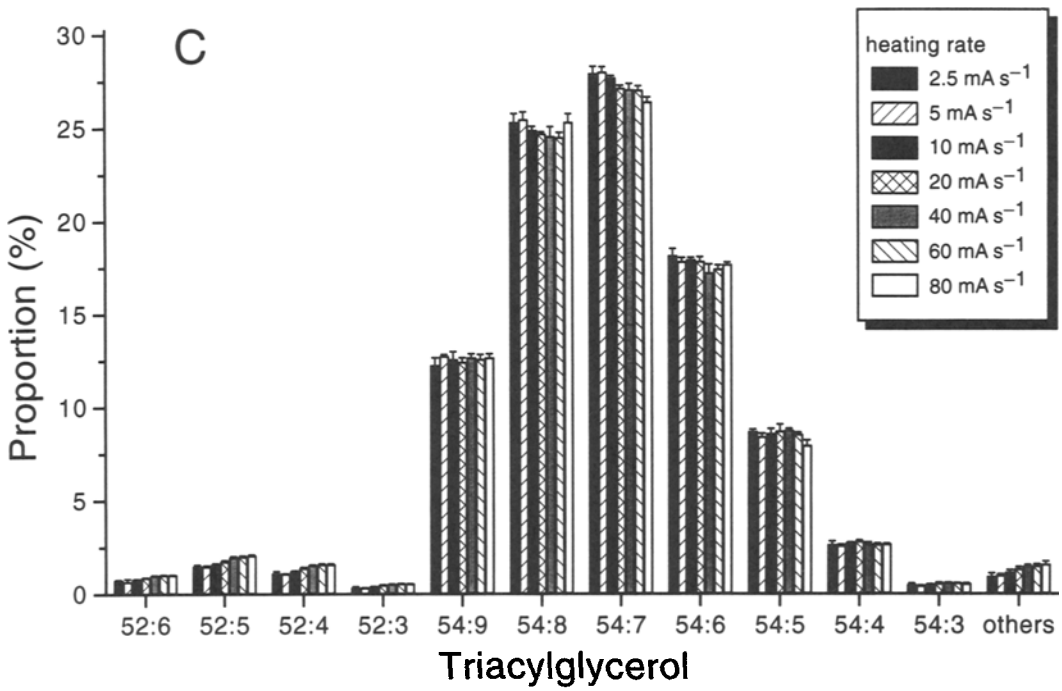
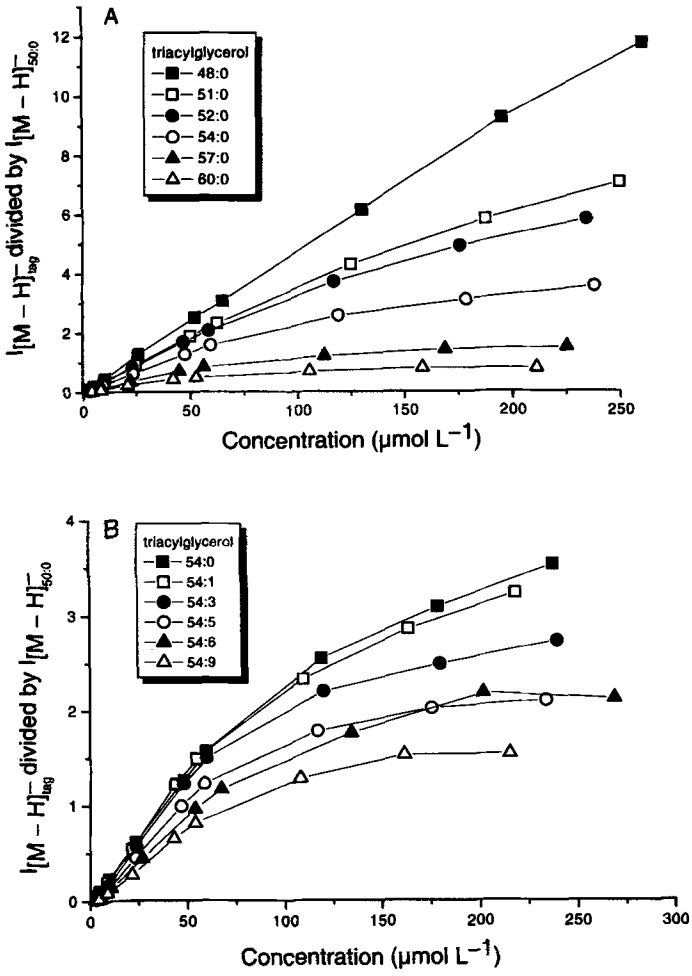


FIG. 3. (continued).



should be approximately the same to prevent mistakes in interpretation of the results caused by concentration differences. Under well-controlled conditions, the method is capable for semiquantitative determination of TAG mixtures of most fats and oils consisting of TAG with 50–56 acyl carbons without the use of correction factors.

Reproducibility of the analysis. The reproducibility of the mass spectrometric determination was studied with a mixture of fifteen TAG standards (mixture B, Table 1, Fig. 6A) and with the TAG isolated from the seeds of raspberry (Fig. 6B). The results are expressed as the mean of four analyses with error bars indicating the standard deviations. The coefficient of variation was typically less than 10% for the components comprising 1% or more of the total amount of TAG. In addition, the reproducibility of the determination from day to day and over longer periods was tested at different points of time. No major changes were observed in the molecular weight distribution of the TAG based on the averaged abundances of $[M - H]^-$ ions. The differences were more obvious with the fifteen-component mixture; nevertheless, reasonable reproducibility of the analysis was achieved in the *m/z* range, which covers the molecular weight area of TAG present in

FIG. 4. Response curves for the $[M - H]^-$ ions of A, the saturated triacylglycerols; and B, the unsaturated triacylglycerols having 54 acyl carbons (reference mixture A, see Table 1) with the addition of 24.7 μmol L^{-1} of *rac*-1,2-distearoyl-3-myristoyl-*sn*-glycerol as internal standard. A volume of 0.5 μL of the sample was applied to the rhenium wire of the direct exposure probe. Reactant gas pressure, 8500 mtorr; and ion source temperature, 200°C. The heating rate of the direct exposure probe was 4 mA s^{-1}; I = intensity; tag = triacylglycerol.

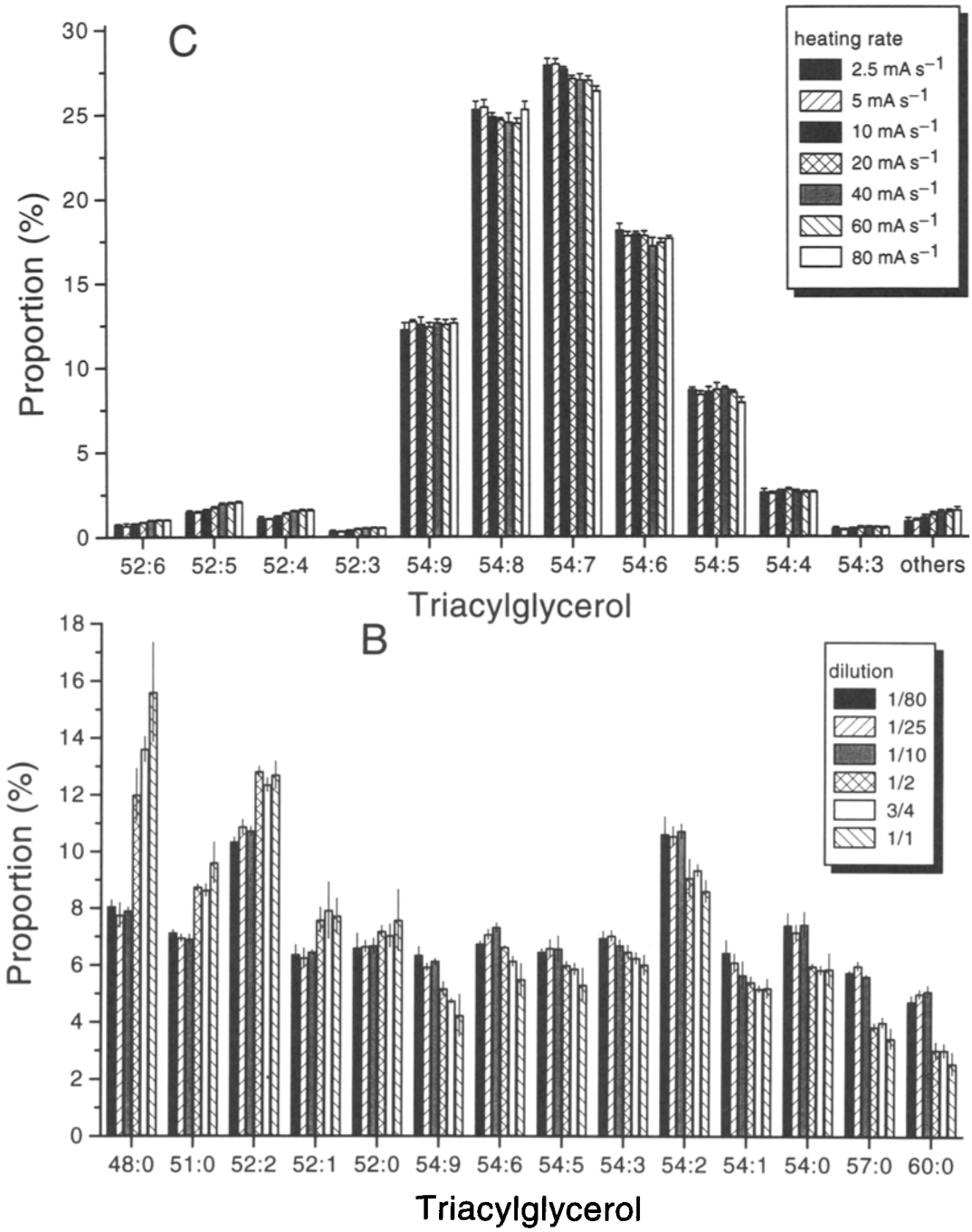


FIG. 5. The effect of concentration on the proportions of triacylglycerols of the reference mixture A (see Table 1) using A, a low (4 mA s^{-1}) and B, a high (80 mA s^{-1}) heating rate of the direct exposure probe. A volume of 0.5 µL of sample was applied to the rhenium wire of the direct exposure probe. The proportions of triacylglycerols are based on the ^{13}C corrected intensities of $[M - H]^-$ ions of four analyses. Reactant gas pressure, 8500 mtorr; and ion source temperature, 200°C.

most seed oils. Minor differences in the distribution may be explained by changes occurring in the sample as well as how the instrument has been tuned.

Mass spectrometric analysis of TAG using ammonia negative-ion CI is a fast and useful method to determine the molecular weight distribution of TAG. Information is obtained on both the number of acyl carbons and double bonds in TAG and the proportions of different molecular weight species. Although we have optimized the analytical conditions for the determination of TAG present in berry seed oils, the method can also be applied to other samples containing mainly TAG with 52, 54, and 56 acyl carbons. The analysis of more complex samples containing either a wide range of TAG with different molecular sizes, such as milk fat, or highly unsaturated

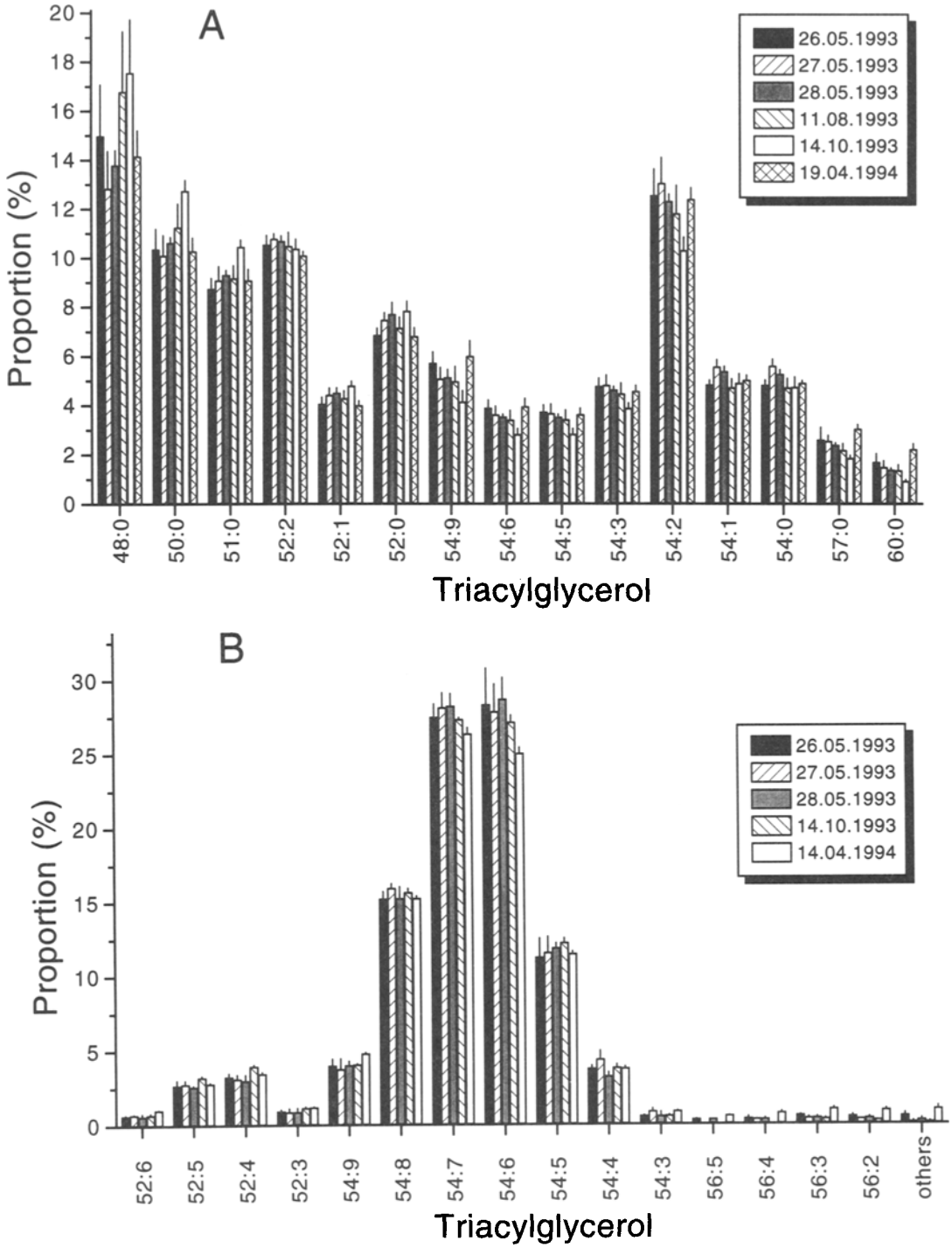


FIG. 6. The repeatability and reproducibility of the mass spectrometric determination for the molecular weight distribution of triacylglycerols: A, reference mixture B (see Table 1), and B, raspberry seed oil. The proportions of triacylglycerols are based on the ^{13}C corrected abundances of $[M - H]^-$ ions of four analyses. Reactant gas pressure, 8500 mtorr; and ion source temperature, 200°C.

components, such as fish oils, may require further optimization of the conditions. In the case of complex samples, it may be advantageous to separate or fractionate the samples, e.g., by chromatography prior to MS determination. This would facilitate the interpretation of the results. Compared with MS, unit resolution of TAG cannot be achieved with either GC or HPLC; GC is not suitable for the analysis of highly unsaturated TAG because of their thermal degradation and polymer-

TABLE 2
Correction Factors for Triacylglycerols in Relation to Tripalmitoylglycerol Using a Slow and Rapid Heating of the Direct Exposure Probe

	Correction factor[a]					
	Dilution[b]					
Triacylglycerol	1/80	1/25	1/10	1/2	3/4	1/1
Slow heating (4 mA s^{-1})						
48:0	1.00	1.00	1.00	1.00	1.00	1.00
51:0	1.22	1.08	1.12	1.25	1.22	1.17
52:0	1.17	1.10	1.06	1.33	1.30	1.27
52:1	1.17	1.12	1.13	1.31	1.33	1.29
52:2	1.49	1.26	1.24	1.39	1.46	1.44
54:0	1.24	0.98	1.14	1.54	1.61	1.63
54:1	1.33	1.09	1.12	1.58	1.68	1.70
54:2	1.43	1.15	1.17	1.45	1.58	1.59
54:3	2.09	1.47	1.38	1.63	1.83	1.93
54:5	3.14	1.85	1.81	1.91	2.22	2.34
54:6	5.04	2.80	2.18	2.21	2.57	2.75
54:9	8.03	4.51	2.85	2.53	2.95	3.18
57:0	1.68	1.36	1.40	2.20	2.61	2.88
60:0	2.22	1.64	1.52	2.65	3.47	4.09
Rapid heating (80 mA s^{-1})						
48:0	1.00	1.00	1.00	1.00	1.00	1.00
51:0	1.08	1.07	1.10	1.32	1.51	1.56
52:0	1.10	1.05	1.07	1.50	1.74	1.85
52:1	1.13	1.11	1.09	1.41	1.53	1.80
52:2	1.14	1.04	1.07	1.37	1.61	1.79
54:0	0.99	0.99	0.97	1.83	2.13	2.43
54:1	1.05	1.06	1.17	1.86	2.20	2.51
54:2	0.94	0.91	0.91	1.64	1.81	2.25
54:3	1.07	1.01	1.09	1.71	2.00	2.39
54:5	1.12	1.05	1.08	1.80	2.08	2.64
54:6	1.23	1.13	1.11	1.86	2.29	2.93
54:9	1.05	1.08	1.07	1.92	2.37	3.05
57:0	1.21	1.12	1.21	2.70	2.94	3.92
60:0	1.38	1.25	1.26	3.20	3.64	4.93

[a]The ^{13}C corrected abundances of $[M-H]^-$ ions were corrected to correspond an equimolar amount of each triacylglycerol before calculating the correction factors as the abundance of $[M-H]^-$ ion of 48:0 divided by the abundance of $[M-H]^-$ ion of the analyte.
[b]Different dilutions (vol_{stock}/vol_{tot}) of the reference mixture A (see Table 1).

ization at the temperatures needed for analysis; and a problem with HPLC is the lack of universal detector without sample discrimination, although light-scattering detectors are useful for many applications. In addition, the mass spectrometric analysis of TAG *via* a direct exposure probe is a very fast method—one analysis takes only 2–3 min. In this study, we have demonstrated that the analysis of TAG, without discrimination according to molecular size or unsaturation, is possible under well controlled mass spectrometric conditions (optimized CI conditions, small enough sample amount). These findings have not yet been verified with other instruments in other laboratories. However, similar results should be achievable elsewhere with comparable instruments after optimization of the analysis of TAG. This technique allows rapid determination of TAG profile, which is very informative in addition to the fatty acid composition of the sample, for example for the needs of quality control in the food industry. Molecular species information could be valuable to improve technological properties of various products or to design products for special purposes, e.g., milk substitutes for infants. This technique may also allow rapid determination of adulterations of various fats and oils, if there are enough differences in the native TAG compositions of the different raw materials.

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COMMUNICATION

Vitamin E Protects Chick Tissues Against *ex vivo* Oxidation of Heme Protein

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ABSTRACT: Protection by dietary vitamin E against *ex vivo* oxidation of heme proteins in liver and heart tissues of chicks was studied. A previously developed assay consisting of deconvoluting spectra obtained from tissue homogenates to measure oxidation of heme proteins proved successful. Compared to liver and heart from chicks fed the basal diet, the tissues from chicks receiving vitamin E exhibited less oxidation.
Lipids 31, 43–46 (1996).

Vitamin E is recognized as the major lipid antioxidant which is important in protecting tissues against oxidative damage and diseases (1). The purposes of this study were to study the protection against homogenate oxidative damage by dietary vitamin E; to test oxidation of heme proteins as a measure of oxidative damage; and to extend studies to another animal, the chick, which utilizes lipids and vitamin E differently than other laboratory animals, such as the rat.

The results of this study show protection by vitamin E of chick liver and heart homogenates against oxidative damage to heme proteins. The usefulness of the heme protein spectra analysis program (HPSAP), a recently developed method of measuring oxidized heme proteins, is confirmed.

MATERIALS AND METHODS

Animals and diets. Twelve male white leghorn day-old chicks were assigned to two groups fed either basal diet (Diet B) or Diet B plus vitamin E (B + E). Diet B, Dyets #13600 (Dyets Inc., Bethlehem, PA) was adequate for growing chicks in all nutrients except vitamin E. Diet B contained 200 g/kg diet soy protein, 4 g/kg DL-methionine, 424 g/kg corn starch, 200 g/kg dextrose, 50 g/kg cellulose, 50 g/kg tocopherol stripped corn oil, 60 g/kg salt mix #236001, 10 g/kg vitamin mix, and 2 g/kg choline bitartrate. For one group of chicks, Diet B was supplemented with 50 IU/kg vitamin E (Diet B + E). The chicks were on the experimental diets for a period of three weeks.

Preparation, oxidative reactions, and spectrophotometric measurement of tissue homogenates. Chicks were decapitated, and nonperfused liver and heart were dissected and frozen immediately. One gram of the frozen tissues was homogenized with 9 mL oxygenated Krebs-Ringer phosphate buffer containing glucose (10 mmol/L, pH 7.4) in a motor-driven tissue homogenizer. One and a half mL of tissue homogenate was incubated at 37°C in a gyratory water bath shaker (New Brunswick Scientific Co., Inc., New Brunswick, NJ) with continuous shaking (180 cycles/min).

After the incubation, 0.6 mL of tissue homogenate was mixed with 0.6 mL of glycerol. The sample was scanned from 500–640 nm, and the absorbance vs. wavelength at 5 nm intervals was automatically recorded by a scan program in a Beckman DU-50 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA).

Absorbance spectra of the tissue heme proteins were analyzed with an HPSAP recently developed in this laboratory (2). The program contains literature values of micromolar extinction coefficients from 500 to 640 nm in 5 nm increments of heme compounds known to exist in liver and heart. The heme protein spectra included in the program are oxyhemoglobin, deoxyhemoglobin, methemoglobin, ferrylhemoglobin, and hemichrome. The corresponding myoglobin derivatives have essentially identical spectra. The program also contains the micromolar extinction coefficients for combined mitochondrial cytochromes and combined microsomal cytochromes. Micromolar extinction coefficients for reduced and oxidized mitochondrial and microsomal cytochromes are calculated according to the ratio of these compounds in chicken liver and heart. The HPSAP also contains a turbidity factor, which previous research has shown to be a linear function of wavelength, with a slope of −1.3 under the experimental conditions used in the study.

To determine micromolar concentrations of heme proteins, absorbance data were entered into the HPSAP. Quantitative determination of the heme compounds in the sample was achieved by a successive approximations fit between the experimental and calculated spectra. The calculated spectrum is based on the assumption that heme compounds closely obey Beer's law. The HPSAP records and subtracts from the absorbance of the sample any contribution due to turbidity in the sample and calculates the mole percent from the micromolar concentration of the individual heme compounds present in the sample.

Statistical analysis. The statistical package SAS (SAS Institute Inc., Cary, NC) was used to analyze all the experimen-

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Abbreviations: HPSAP, heme protein spectra analysis program; SSD, sum of squared differences.

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tal data. When significant F-values were obtained using analysis of variance, Duncan's least significant difference procedure was used to determine significant differences ($P < 0.05$) between treatment means.

The sum of squared differences (SSD) was used to evaluate the reliability of HPSAP in analyzing heme proteins in tissue homogenates by reconciling the experimental spectrum (as recorded by the spectrophotometer) and the calculated spectrum (determined with HPSAP). The SSD is calculated as follows: SSD = Σ (calculated absorbance − experimental absorbance) 2. The best fit is indicated as the SSD approaches zero.

RESULTS

Figures 1 and 2 show the data points for representative experimental spectra obtained at timed intervals during the oxidation of liver and heart homogenates from chicks fed basal diet (B) or basal diet supplemented with vitamin E (B + E). The experimental spectra typically have an absorbance of 1 of background turbidity due to absorbances of homogenate components other than the heme proteins. These experimental spectra are subjected to analytical deconvolution with HPSAP. Table 1 shows some representative analyses with HPSAP. The heme protein concentrations are calculated in μM, but because there are different concentrations in each sample from nonperfused tissue, the concentrations of individual and total heme proteins are normalized to mole percent.

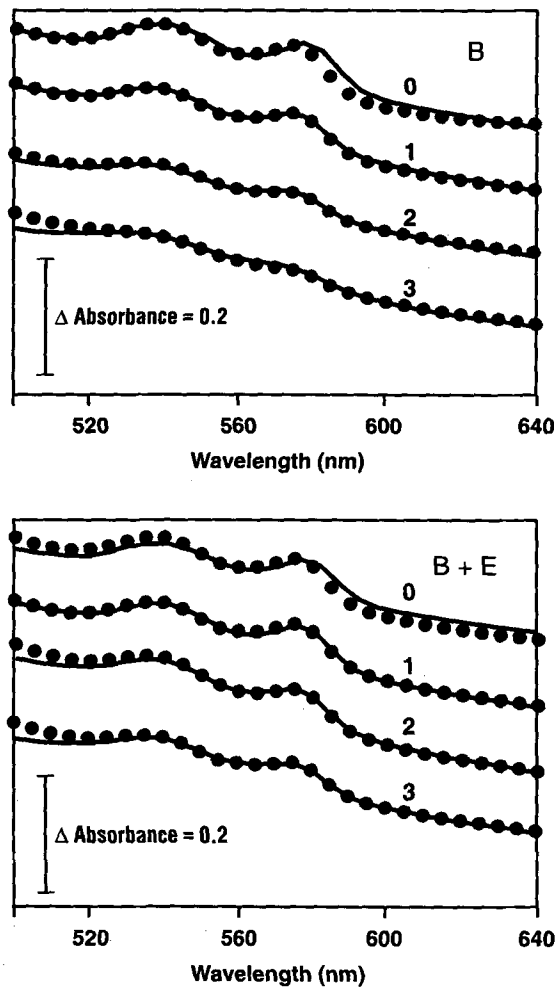


FIG. 1. Visible spectra of heme proteins in liver homogenates incubated at 37°C; 0 h (0); 1 h (1); 2 h (2); 3 h (3); •••, experimental data for spectra; ——, spectra calculated *via* heme protein spectra analysis program. Liver homogenates were from chicks fed basal Diet (B) or basal diet + vitamin E (B + E).

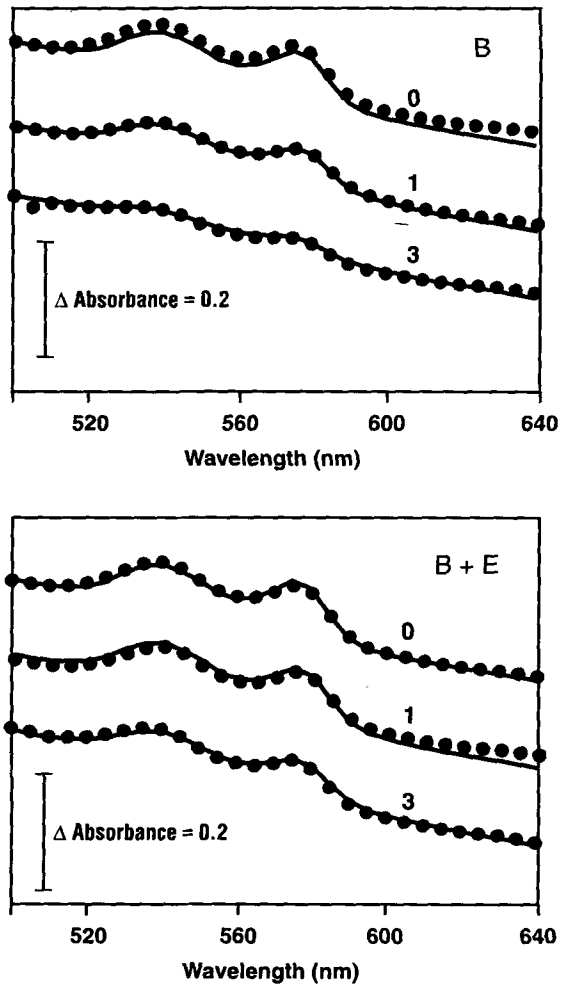


FIG. 2. Visible spectra of heme proteins in heart homogenates incubated at 37°C; 0 h (0); 1 h (1); 3 h (3); •••, experimental data for spectra; ——, spectra calculated *via* heme protein spectra analysis program. Heart homogenates were from chicks fed basal diet (B) or basal diet + vitamin E (B + E).

The low SSD values confirm the very good fits between the experimental spectral data and the calculated spectra, representing the sum of the spectra of the calculated heme proteins as shown in Table 1.

To further examine the correspondence between the experimental spectral data and the calculated spectra, the calculated spectra are superimposed on the experimental data as shown in Figures 1 and 2. The SSD was calculated for 90 spectra an-

TABLE 1
Heme Proteins Present in Liver and Heart Homogenates After Oxidation as Calculated with HPSAP[a]

	Liver				Heart			
	Diet B		Diet B + E		Diet B		Diet B + E	
	0 h	3 h	0 h	3 h	0 h	3 h	0 h	3 h
Oxyhemoglobin or/and oxymyoglobin (mole %)	100	14	100	44	100	35	100	60
Methemoglobin or/and metmyoglobin (mole %)	0	35	0	17	0	38	0	18
Hemichrome (mole %)	0	51	0	39	0	27	0	22
SSD	0.0034	0.012	0.0025	0.013	0.0040	0.0018	0.0020	0.0008
Total heme protein concentration (mM)	14	—	14	—	13	—	15	—

[a]HPSAP, heme protein spectra analysis program; Diet B, basal diet; Diet B + E, basal diet + vitamin E; SSD, sum of squared differences.

alyzed during the course of this work, and SSD values ranged from 0.00029 to 0.0170. Eighty percent of the SSD were below 0.0035, and 90% were less than 0.0055.

The experimental results of incubating liver and heart homogenates under oxidizing conditions can be seen in Figures 1 and 2 and Table 1. In the nonperfused liver, oxyhemoglobin from the blood is the dominant heme protein at 0 h. In the nonperfused heart, oxyhemoglobin and oxymyoglobin are the dominant heme proteins at 0 h. The spectra of both oxyhemoglobin and oxymyoglobin have the characteristic peaks at 545 and 577 nm. As the heme proteins in the homogenates oxidize with increasing incubation time, the spectra in Figures 1 and 2 and the analyses in Table 1 show the progressive formation of methemoglobin–metmyoglobin and hemichromes which have flatter spectra than oxyhemoglobin–oxymyoglobin.

The concentrations of α-tocopherol in liver tissue from chicks fed Diets B and B + E were <4 and 28 ± 5 μg α-tocopherol/g tissue, respectively. The concentrations of α-tocopherol in heart tissue from chicks fed Diets B and B + E were <4 and 18 μg α-tocopherol/g tissue, respectively (3).

The calculated heme proteins, for which Table 1 shows representative data, were totaled and averaged in Table 2 to show the mole percent oxidized heme proteins as a function of incubation time and vitamin E status. Table 2 records the decreased mole percent of oxidized heme proteins of liver and heart from chicks fed the Diet B + E compared to oxidized heme proteins from chicks fed only basal diet. These antioxidant effects were highly significant for liver homogenates ($P < 0.0006$) and heart homogenates ($P < 0.0014$). It is interesting to compare this data for the chick to similar data for the rat. The increase in percent oxidized heme proteins for liver from chicks on the basal diet compared to Diet B + E correlated with similar data from rats (4) with r = 0.99 ($P < 0.01$).

TABLE 2
Effects of Vitamin E on Total Oxidized Heme Proteins in Liver and Heart Homogenates

		Mole % oxidized heme proteins	
Tissue	Incubation time (h)	Basal diet	Basal diet + vitamin E
Liver	0	0	0
	1	44.4 ± 9.3	18.5 ± 11.0
	2	50.7.± 9.4	36.0 ± 20.7
	3	73.3 ± 11.9	43.2 ± 27.3
Heart	0	0	0
	1	27.7 ± 11.1	18.2 ± 15.0
	3	51.9 ± 11.4	29.6 ± 7.3

DISCUSSION

To understand damaging biological oxidative processes, it is necessary to quantify damage to proteins, lipids, and other cellular components. The quantitation of mixtures of heme proteins in biological systems has long been a problem. This is evidenced by the many spectra presented in the literature without quantitation. HPSAP has been developed (2) as a tool for multicomponent analysis using a full spectrum method. As shown in this research on chick tissues, this method is a powerful and versatile tool for analyzing heme proteins and their oxidative products. SSD is an appropriate statistical technique of evaluating the HPSAP. The low SSD values demonstrate that HPSAP is an accurate method for fitting spectra and thus for measuring oxidation of heme proteins.

HPSAP has been applied to spectra of mixtures of heme proteins from the literature including reflectance spectra of human skin *in vivo* and to laboratory preparations of these mixtures, including the complex spectra presented by tissue slices and homogenates, with equally good results (2).

This method is applicable to investigations of oxidative damage to animal tissues by toxic oxidants (4). HPSAP is also useful in studies of the protective effects of nutrient lipid antioxidants against oxidative damage to tissues (5,6).

Dietary vitamin E is known to be deposited in liver and heart of many animals (7). Vitamin E protects tissues against the major deteriorative processes, mainly lipid peroxidation. In a study of vitamin E protection against heme protein oxidation and lipid peroxidation in chicken liver slices, lipid peroxidation as measured by the production of thiobarbituric acid reacting substances has been found to correlate with heme protein oxidation (8).

The present study provides information on the protection by dietary vitamin E in the chick of liver and heart homogenates against heme protein oxidation. Because there is little information in the literature on comparative rates of tissue homogenate oxidations, it is significant to find the strong correlation between the rates of formation of oxidized heme proteins in chick and rat liver.

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Protection by Multiple Antioxidants Against Lipid Peroxidation in Rat Liver Homogenate

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ABSTRACT: The purpose of this study was to test the hypothesis that multiple antioxygenic nutrients provide increased protection against lipid peroxidative damage to rat liver. Rats were fed diets (i) deficient in vitamin E and selenium (Diet 1), (ii) supplemented with vitamin E and selenium (Diet 2), (iii) supplemented with (ii) and in addition trolox C, *N*-acetylcysteine, coenzyme Q_0, and (+)-catechin (Diet 3), or (iv) supplemented with (iii) and in addition β-carotene, ascorbic acid palmitate, canthaxanthin, and coenzyme Q_{10} (Diet 4). Liver homogenates were obtained from three rats fed each of the diets for six weeks and were incubated at 37°C up to two hours with and without exogenous tertiary-butyl hydroperoxide (TBHP) or Cu^{2+}. Lipid peroxidation was determined by measurement of thiobarbituric acid substances. Diets 2 and 3 significantly protected against *in vivo* hepatic lipid peroxidation, and this protection was augmented by Diet 4. Diets 2, 3, and 4 were protective against mild oxidation induced by TBHP or Cu^{2+}. During incubations with exogenous TBHP and Cu^{2+}, there were only small differences between diets supplemented with antioxidants in inhibition of lipid peroxidation, indicating that diets supplemented with vitamin E and selenium (Diet 2) may have provided the maximal protection for liver. The possible mechanisms of protection provided by multiple antioxidants in diets were discussed. Protection by multiple antioxidants against lipid peroxidation may translate to prevention of peroxidative damage to human tissue, a factor in human disease.
Lipids 31, 47–50 (1996).

Lipid peroxidation, the major free radical reaction in cellular membranes, can cause serious oxidative damage to animal cells and tissues. Reactive oxygen species generated from lipid peroxidation rapidly interact with proteins, enzymes, and DNA molecules. Consequences of such interactions are involved in the development of various disease states such as atherosclerosis, cardiovascular disease, degenerative diseases, and cancer (1,2).

Vitamin E, ascorbic acid, thiol compounds, carotenoids, coenzyme Q, and flavonoids are potent antioxidants that can effectively inhibit lipid peroxidation and thus protect tissues against oxidative damage (3–5). Supplementary dietary antioxidants have shown beneficial effects in prevention of certain diseases. For example, some epidemiological studies have shown that dietary intake of high doses of vitamin E is associated with a reduced risk of heart disease (6).

If individual antioxidants can effectively inhibit lipid peroxidation both *in vitro* and *in vivo* as suggested by the above studies, it would be expected that a system containing multiple antioxidants could be more effective in protecting tissues against oxidative damage. First, multiple antioxidants in a system would provide complementary antioxygenic functions. A system containing both fat-soluble and water-soluble antioxidants will be more effective than a system containing only fat-soluble or water-soluble antioxidants because the former is capable of intercepting reactive oxygen species from both aqueous and lipid phases (3). Second, synergistic interaction could strengthen the functions of individual antioxidants. The antioxygenic functions of vitamin E can be augmented when ascorbic acid is present due to the participation of ascorbic acid in the regeneration of vitamin E (7). Third, addition of multiple antioxidants to diets would increase the total quantity of antioxidants. Recently, there have been studies of the effects of multiple antioxidants in cellular defense against oxidative damage (8–10).

The purpose of this study was to test the concept that diets containing multiple antioxidants would be more effective in protection of liver against lipid peroxidation. The diets were supplemented with vitamin E, trolox C (the water-soluble vitamin E analog), selenium, a thiol compound, carotenoids, coenzyme Q, ascorbic acid palmitate, and (+)-catechin, a flavonoid. There are three primary reasons for choosing these antioxidants. These antioxidants have shown strong protection against lipid peroxidation at least in *in vitro* systems. In addition, most of these antioxidants are present in human foods so that the results from this study would be applicable to people. Finally, effects of combinations of these dietary antioxidants have not been studied thoroughly. In this study, rats were fed either an antioxidant-deficient diet or diets containing multiple antioxidants for six weeks. To test the effectiveness of diets supplemented with antioxidants in protection against lipid peroxidation, liver homogenates were incubated at 37°C with and without the addition of the exogenous prooxidants tertiary-butyl hydroperoxide (TBHP) and Cu^{2+}.

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Abbreviations: TBARS, thiobarbituric acid-reactive substances; TBHP, tertiary-butyl hydroperoxide.

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TBHP is a representative toxic hydroperoxide, a class of compounds constantly formed in small amounts in normal metabolism. Cu^{2+} is a trace element which can initiate lipid peroxidation *in vivo*. Lipid peroxidation was measured as thiobarbituric acid-reactive substances (TBARS). The results from spontaneous lipid peroxidation provided information on the protection by multiple antioxidants against oxidative damage under conditions close to the *in vivo* situation. The results from lipid peroxidation induced by TBHP or Cu^{2+} showed the degree of effectiveness of multiple antioxidants in inhibition of lipid peroxidation initiated by specific prooxidants.

MATERIALS AND METHODS

Chemicals. The antioxidants and chemicals used in this study were (+)-α-tocopherol acid succinate (1210 I.U./g), sodium selenite (Alfa Inorganics, Beverly, MA), trolox C (Aldrich Chemical Co., Milwaukee, WI), coenzyme Q_0, coenzyme Q_{10}, (+)-catechin, dimethyl sulfoxide, β-carotene, ascorbic acid palmitate, *N*-acetylcysteine (Sigma Chemical, St. Louis, MO), canthaxanthin (Roche Vitamin and Fine Chemicals, Nutley, NJ), TBHP (Polysciences, Inc., Warrington, PA), and cupric chloride ($CuCl_2$) (Merck & Co. Inc., Rahway, NJ).

Animals and diets. Male Sprague-Dawley rats (Bantin & Kingman, Fremont, CA), weighing 40–60 g, were adapted for two days before being fed experimental diets. The basal diet (Diet 1) was deficient in vitamin E and selenium with 10% tocopherol stripped corn oil (Teklad Test Diet No. TD 77068, mineral mix No. 170911; Teklad Test Diets, Madison, WI). Animals were housed according to National Institutes of Health guidelines and had free access to deionized water and diets. Diets supplemented with antioxidants were divided into three groups: Diet 2, containing 25 mg vitamin E/kg diet and 0.3 mg selenium/kg diet; Diet 3, containing vitamin E, selenium, and the water-soluble antioxidants 50 mg trolox C/kg diet, 200 mg *N*-acetylcysteine/kg diet, 100 mg coenzyme Q_0/kg diet; and Diet 4, containing vitamin E, selenium, the water-soluble antioxidants and the fat-soluble antioxidants 30 mg coenzyme Q_{10}/kg diet, 45 mg β-carotene/kg diet, 45 mg canthaxanthin/kg diet, 100 mg ascorbic acid palmitate/kg diet. The (+)-catechin/kg diet (100 mg) was added to Diets 3 and 4 as a representative flavonoid. The rats were fed the experimental diets along with distilled water for six weeks.

Preparation of liver homogenates. The rats were decapitated, and livers were immediately dissected and immersed in ice-cold Krebs-Ringer phosphate buffer (pH 7.4). Livers were blotted with filter papers and stored at −22°C. Frozen livers were cut into 0.5 cm^3 cubes, and homogenates were prepared by homogenizing 1 g of liver with 9 mL of oxygenated buffer containing glucose (10 mmol, pH 7.4). A motor-driven tissue homogenizer was used.

Oxidation of homogenates and measurement of TBARS. Oxidation of tissue homogenates and measurement of TBARS followed the method described by Tappel and Zalkin (11) with some modifications. Two mL of homogenates were transferred to a 10-mL glass serum bottle containing 3 mL of oxygenated 0.9% NaCl. TBHP or Cu^{2+} for a final concentration of 0.1–1.0 mM, was added to the serum bottle immediately before the reaction began. The homogenates were incubated in the water-bath shaker at 37°C with continuous shaking (180 cycles/min). After incubation, the bottles were cooled with dry ice. Homogenates (2.5 mL) were transferred to a 10-mL centrifuge tube containing 2.5 mL of 10% trichloroacetic acid. The homogenates were centrifuged at 3000 rpm for 2 min at 4°C. After centrifugation, 4 mL of supernatant were reacted with 1.25 mL of 0.75% thiobarbituric acid (freshly prepared) at 100°C for 10 min. The resulting pink color was measured at 530 nm. TBARS were expressed as malondialdehyde equivalents per gram tissue.

The statistical package SAS (SAS Institute Inc., Cary, NC) was used to analyze all data. When significant F-values were obtained using analysis of variance, Duncan's least square means procedure was used to determine the significant difference ($P \leq 0.05$) between treatment means. Results were expressed as means ± standard deviation.

RESULTS

Time course of protection by antioxidants with or without prooxidants. Dietary treatments had no effect on the weight gained by the animals. No exogenous prooxidants were introduced to the incubation vessels during spontaneous lipid peroxidation (time 0). The concentrations of TBARS in liver homogenate from rats fed an antioxidant-deficient diet (Diet 1) rose substantially as incubation time increased (Fig. 1). Addi-

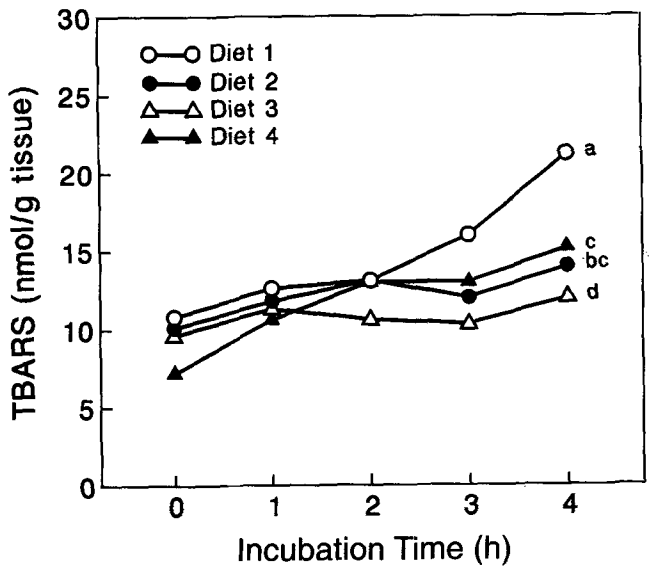


FIG. 1. Time course of spontaneous lipid peroxidation in liver homogenate showing protection by antioxidants; Diet 1 (basal diet). Liver homogenates were incubated at 37°C. Five timed measurements were taken from homogenates obtained from each rat. The values are expressed as mean for three rats. Curves not sharing a common letter are significantly different from each other at a 95% confidence level. The amounts of thiobarbituric acid-reacting substances (TBARS) are denoted by the alphabetic order with "a" as the largest and "d" as the smallest.

tional vitamin E and selenium (Diet 2) inhibited the formation of TBARS, particularly during the relatively long periods of incubation (3 and 4 h). Diets enriched with water-soluble antioxygenic nutrients (Diet 3) significantly augmented protection against lipid peroxidation provided by Diet 2. In the *in vivo* situation (incubation time 0), Diet 4, enriched with both water- and fat-soluble antioxidants, provided significantly better protection against lipid peroxidation than either Diets 2 or 3. As incubation proceeded, however, Diet 4 protected liver as effectively as Diet 2, but less effectively than Diet 3.

In the reactions induced by either TBHP or Cu^{2+}, dietary supplementation by vitamin E and selenium significantly lowered the production of TBARS only after 2 h and Cu^{2+} after 0.5 and 1.0 h (Fig. 2). Feeding Diet 4 markedly decreased *in vivo* lipid peroxidation compared to Diets 2 and 3. With additional antioxidants as incubation time progressed, there was no further decrease in the production of TBARS.

Protection by antioxidants against various concentrations of prooxidants. As the concentration of prooxidants increased in the incubated homogenates from the four dietary groups, the production of TBARS rose correspondingly in the systems induced by TBHP or Cu^{2+} (Fig. 3). When Cu^{2+} induced lipid peroxidation, the addition of vitamin E and selenium to the basal diet (Diet 2) suppressed the production of TBARS through 1.0 mM Cu^{2+}, and in the reaction initiated by TBHP Diet 2 suppressed TBARS through the 0.8 mM level of the inducer. Diet enriched with water-soluble antioxidants (Diet 3) improved the protection provided by Diet 2 for liver incubated with 0.8 and 1 mM TBHP but not with Cu^{2+}. In contrast, diet enriched with a full complement of antioxidants (Diet 4) appeared to offer no greater protection than that provided by Diet 2 for liver incubated with various concentrations of TBHP or Cu^{2+}.

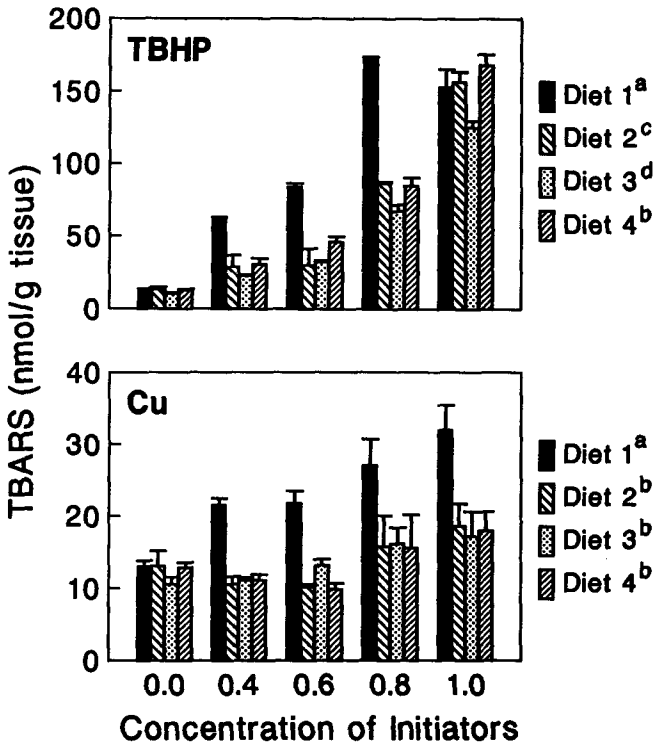


FIG. 3. Protection by antioxidants against lipid peroxidation of liver tissues induced by concentrations of TBHP or Cu^{2+} ranging from 0 to 1.0 mM. Liver homogenates were incubated at 37°C for 2 h. Five measurements were taken from homogenates obtained from each rat. The values are expressed as mean for three rats. TBARS of the concentration sequence of initiators not sharing common superscript letters are significantly different from each other at a 95% confidence level. The amount of TBARS is denoted by alphabetic order with "a" as the largest and "d" as the smallest. See Figures 1 and 2 for abbreviations.

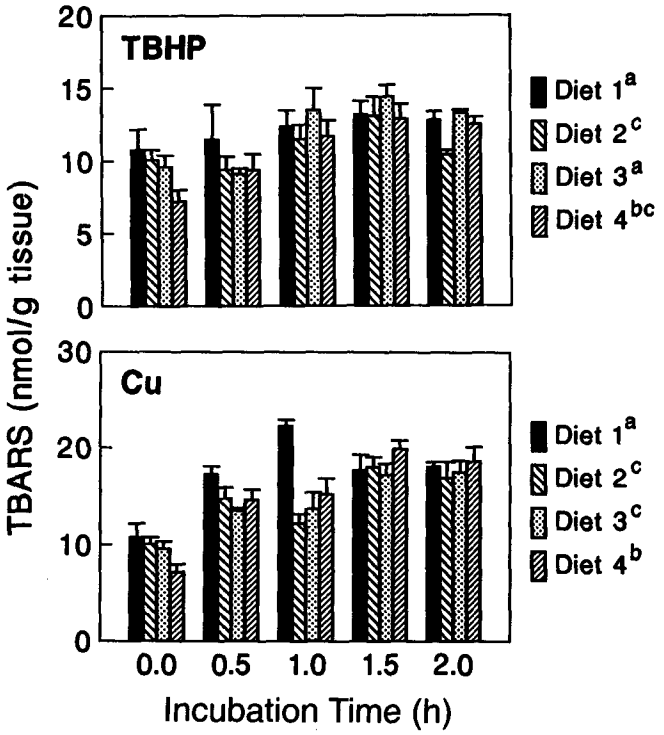


FIG. 2. Time-course study of the protection by antioxidants against lipid peroxidation of liver homogenates induced by tertiary-butyl hydroperoxide (TBHP) or Cu^{2+}. Liver homogenates were incubated at 37°C with 0.1 mM TBHP or Cu^{2+}. Five timed measurements were taken from homogenates obtained from each rat. The values are expressed as mean for three rats. The time sequence of TBARS not sharing a common superscript letter are significantly different from each other at a 95% confidence level. The amount of TBARS are denoted by the alphabetic order with "a" as the largest and "e" as the smallest. See Figure 1 for other abbreviation.

DISCUSSION

The antioxidants were chosen for this experimental study because there is a considerable amount of biochemical knowledge about each of them in the scientific literature: vitamin E, selenium, ascorbic acid palpitate, acetylcysteine, and coenzyme Q (1); trolox-C (12); β-carotene (2); and (+)-catechin (10). Previous studies have analyzed the deposition in vari-

ous tissues following dietary supplementation by vitamin E and selenium (8) and coenzyme Q (13), and β-carotene (14).

This research, using many antioxidants, is exploratory. Discussion of results is more empirical than mechanistic. The most important observation (Fig. 1) is that Diet 4 containing the most diverse antioxidants effected the lowest initial level of TBARS in the *in vivo* situation (incubation at 0 min). The superior performance of Diet 3 from 2–4 h and Diet 4 at 0 time may be attributed to the diversity of the antioxidants present in the diet, the level of concentration of the antioxidants, and synergistic effects among the antioxidants.

Antioxidants can inhibit lipid peroxidation either by reducing the rate of chain initiation (preventive antioxidants) or trapping free radicals (chain-breaking antioxidants) or both modes of action (1). Carotenoids, vitamin E, coenzyme Q, and flavonoids are excellent preventive antioxidants, due to their unusual ability to quench singlet oxygen (15–17). Vitamin E, ascorbate, and trolox C are potent chain-breaking antioxidants, due to their large rate constants for the reaction with peroxyl radicals. Apparently, the combination of preventive and chain-breaking dietary antioxidants, such as Diets 3 and 4, strengthens the protective effect against lipid peroxidation provided by Diet 2 that was supplemented with only vitamin E and selenium.

One important feature of Diets 3 and 4 is that they contain both lipid-soluble and water-soluble antioxidants. Even though lipid peroxidation occurs in the lipid phase, mainly in membranes, it can be accelerated by the attack from aqueous peroxyl radicals. Water-soluble antioxidants thus can inhibit lipid peroxidation by interacting with aqueous free radicals (1). For example, experiments have shown that ascorbate is the most effective antioxidant against lipid peroxidation in human blood plasma (17). In this study, the presence of both water- and lipid-soluble antioxidants in the diet also shows significant improvement of protection against *in vivo* lipid peroxidation compared to the diet supplemented only with water-soluble antioxidants.

During incubations with exogenous TBHP or Cu^{2+}, Diets 3 and 4 showed augmented protection over that of Diet 2 against *in vivo* lipid peroxidation in liver (Fig. 2), confirming the inhibition of *in vivo* spontaneous lipid peroxidation shown by Diets 3 and 4 (Fig. 1). With increased incubation times and concentrations of initiators, vitamin E and selenium (Diet 2) may have provided maximal protection against lipid peroxidation induced by TBHP and Cu^{2+} (Figs. 2 and 3). Further increase of quantity and variety of antioxidants offered very little or no improvement.

The results of the present study may be applicable to multiantioxidant protection of tissue and foods against oxidative damage.

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Dietary Fat and Neural Development

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The concept that dietary fats are capable of not only altering the polyunsaturated fatty acid (PUFA) composition of neural tissue, but also can result in altered neural function is relatively new, and it is exciting research attention worldwide. For this reason we offered to organize a one-day symposium during the AOCS Annual Meeting & Expo at the Henry B. Gonzalez Convention Center in San Antonio, Texas, which took place May 7–11, 1995. This symposium was entitled "Dietary Fat and Neural Development." The meeting was designed to bring together the leading workers in the field under the auspices of a learned society (most meetings on this topic are organized by individual formula companies) in an environment that allowed for frank and open discussion. The format of the meeting was based on that developed by the organizers in 1992 at the 3rd International Congress of EFA and Eicosanoids, the proceedings of which were published by AOCS Press in 1993. The topics were organized into three main themes—animal studies, PUFA metabolism in humans, and randomized clinical trials involving human infants. Each of the themes was presented as three 20-minute talks followed by at least 40 minutes of discussion. Both the general topic and the discussion-based format seemed to appeal to participants, and despite this event being held on the final day of the AOCS meeting, the room was packed all day and discussion was intense. The level of interest was such that a whole meeting devoted to the difficulties in carrying out research in this area is being sponsored by AOCS in Barcelona, November 7–9, 1996, under the title "PUFA in Infant Nutrition: Consensus and Controversies."

The present issue of *Lipids* contains the papers presented at the symposium in San Antonio and includes reviews and original research articles. We wish to thank Dr. Howard R. Knapp, Editor-in-Chief of *Lipids*, for reviewing the papers and for his careful attention to detail. We are pleased that the papers from this symposium have appeared in print in less than nine months from the date of the meeting. Finally, we would like to thank AOCS, Wyeth-Ayerst, Mead Johnson, Martek Biosciences, Nestle, and Ross Laboratories for their support.

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Retinal Fatty Acids of Piglets Fed Docosahexaenoic and Arachidonic Acids from Microbial Sources[1]

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ABSTRACT: Docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6) serve important roles in perinatal visual and neural development. A neonatal pig model was used to determine if dietary supplementation with DHA and AA at slightly greater concentrations than normally found in human milk would influence fatty acid accretion in retina. One-day-old piglets were assigned to one of four diets (n = 5/group): (i) STD, standard diet containing fat similar to infant formula; (ii) STD + DHA, 0.7% of fatty acids as DHA; (iii) STD + AA, 0.9% as AA; and (iv) STD + BOTH, 0.8% as DHA plus 1.0% as AA. After 25 d, fatty acids in retina phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were determined. Supplementation with DHA resulted in approximately twofold increases ($P < 0.05$) in PC–DHA (4.88% in STD vs. 10.03% in STD + DHA and 9.47% in STD + BOTH). Similarly, AA supplementation increased PC–AA 1.3–1.4-fold (4.47% in STD vs. 6.19% in STD + AA and 5.70% in STD + BOTH). For PE, supplementation with either fatty acid or in combination resulted in no significant increases, except for a 1.2-fold increase in DHA for STD + BOTH (32.66%) vs. STD (28.38%). Thus, PC responded to dietary supplementation, with addition of DHA, AA, or BOTH, resulting in increases in respective fatty acids; PE was less responsive, with only STD + BOTH resulting in increased DHA. No significant competition between DHA and AA in incorporation into phospholipids was observed. In conclusion, consumption of a combination of DHA and AA by neonatal pigs supported accretion of DHA in retina phospholipids, while simultaneously supplying the AA necessary for membrane phospholipids and eicosanoid biosynthesis.

Lipids 31, 53–59 (1996).

Docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6) serve important roles in neural development in the perinatal period (1). Retina membrane phospholipids, particularly the phosphatidylethanolamine (PE) fraction, contain large amounts of DHA (2,3). Dietary deficiency in n-3 fatty acids is associated with visual abnormalities in rats (4), in nonhuman primates (5–7), in preterm infants (8–11), and in formula-fed term infants (12). During the perinatal period, AA accumulates in phospholipids and plays a vital role as a membrane component (1), as an eicosanoid precursor, and perhaps as a growth factor (13–16). Both DHA and AA are supplied to the developing infant by human breast milk. The concentration of AA is relatively constant in breast milk at about 0.5% of total fatty acids, whereas DHA concentrations vary from 0.2 to 1.9% of total fatty acids depending on the n-3 fatty acid content of the maternal diet (17–20). The higher concentrations are seen only in populations consuming large amounts of fish (21), or in lactating women whose diets are supplemented with fish oil (22). Typically, DHA concentrations would range from 0.2 to 0.6% of total fatty acids (23). Conventional infant formulas do not contain either of these long-chain polyunsaturated fatty acids.

In fully developed animals, the need for DHA and AA can be supplied by their shorter-chain precursors, α-linolenic acid (LNA, 18:3n-3) and linoleic acid (LA, 18:2n-6). However, in the preterm infant and perhaps in the term infant, formula containing only the shorter-chain precursors may be inadequate to meet the increased demands for long-chain polyunsaturated fatty acids with a chain length of 20 or more carbons (24–27). Various sources of preformed n-3 and n-6 polyunsaturated fatty acids have been added recently to some infant formulas. These include borage oil, evening primrose oil, and black currant seed oil as sources of γ-linolenic acid, egg yolk lipid as a source of AA, and marine lipids as a source of DHA (28). Microbial oils as concentrated sources of DHA and AA are currently available, but their efficacy has not been fully documented. Thus, in the current experiment, a neonatal pig model was used to determine if dietary supplementation with DHA and AA at concentrations only slightly greater than that found in human milk would influence fatty acid accretion in retina phospholipids.

MATERIALS AND METHODS

Animals and diets. Twenty-five male cross-bred (Yorkshire ×

[1]Based on a presentation at the AOCS Annual Meeting & Expo in San Antonio, Texas, May 7–11, 1995.

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; FAME, fatty acid methyl ester; LA, linoleic acid; LNA, α-linolenic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SOW, a naturally-reared group fed sow milk; STD, a standard diet containing a fat blend similar to that used in some commercial infant formulas; STD + AA, standard diet supplemented with AA; STD + BOTH, standard diet supplemented with both DHA and AA; STD + DHA, standard diet supplemented with DHA.

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Landrace sows bred to Duroc boars) piglets, weighing at least 1.36 kg at birth, were obtained from the Swine Nutrition Unit, Alabama Agricultural Experiment Station, Auburn University (Auburn, AL). At one day of age, one piglet from each of five litters was assigned randomly to one of four dietary treatments (n = 5/group) for 25 d: (i) STD, a standard diet containing a fat blend similar to that used in some commercial infant formulas; (ii) STD + DHA, standard diet modified to contain 0.7% of total fatty acids as DHA; (iii) STD + AA, standard diet modified to contain 0.9% of total fatty acids as AA; and (iv) STD + BOTH, standard diet modified to contain 0.8% of total fatty acids as DHA and 1.0% as AA. In addition, one piglet from each of the five litters was naturally-reared and was fed sow milk (SOW). The fatty acid composition of the experimental formulas and the sow milk as analyzed is shown in Table 1. The number of piglets remaining in each litter after allocation to formula-fed groups was adjusted by cross-fostering, if necessary, to maintain a constant litter size of five.

The piglets were allowed to suckle for 24 h after birth. Iron dextran injections were given on day 1, but all vaccinations were withheld. All piglets in the four formula-fed groups were housed at the Auburn University School of Veterinary Medicine in stainless-steel cages equipped with heat lamps. From day 1 to day 25, the piglets in the four formula-fed groups were bottle-fed a skim milk-based, sow-milk replacer formula (Pet Ag Inc., Elgin, IL) modified to contain either AA or DHA. Formula fat blends were adjusted to maintain relatively constant amounts of all fatty acids except AA and DHA. All formulas had an LA/LNA ratio of 9:1 (Table 1). Dietary antioxidant content, including β-carotene, was equalized for all formula-fed groups. During the first week, the piglets were fed to appetite five times a day, and after that four times a day. Feed consumption was recorded at each feeding.

The diet of the sows used for the naturally-reared group was a corn/soybean meal diet typically used by commercial swine producers in the United States and formulated to meet National Research Council (NRC) requirements for lactating sows (29). The sow diet was low in fat (2.8% by weight) and contained 1.54% 18:2n-6 and 0.03% 18:3n-3, with an LA/LNA ratio of 50:1. Milk collected from each of the five sows on day 12 of lactation had a LA/LNA ratio of 22:1 and a DHA content of 0.04% of total fatty acids (Table 1). The AA content of the sow milk was 0.7% of total fatty acids.

TABLE 1
Fatty Acid Composition of Experimental Diets

	Treatment diets[a] (% of total fatty acids)				
Fatty acid	STD	STD + DHA	STD + AA	STD + BOTH	SOW
<18C	36.7	38.3	37.5	37.8	52.0
18:0	6.8	6.8	7.5	6.6	3.8
18:1n-9	39.7	37.7	38.0	37.0	28.1
18:1n-7	1.1	1.1	1.2	1.1	1.7
18:2n-6	12.8	12.6	12.1	12.7	12.7
18:3n-3	1.4	1.4	1.3	1.4	0.6
20:0	0.2	0.2	0.2	0.3	—
20:1n-9	0.2	0.2	0.2	0.2	—
20:4n-6	—	—	0.9	1.0	0.7
22:0	0.2	0.2	0.2	0.2	—
22:6n-3	—	0.7	—	0.8	0.04

[a]Fatty acid composition of the standard diet with fatty acid composition similar to that of some current infant formulas (STD); STD + DHA, docosahexaenoic acid-supplemented; STD + AA, arachidonic acid-supplemented; STD + BOTH, DHA-and-AA supplemented; SOW, naturally-reared group fed sow milk.

Weight gain of the piglets was monitored every four days. All procedures involving animals were approved by the Auburn University Institutional Animal Care and Use Committee.

The pigs were euthanized after the 25-d feeding period. Retinas were removed, rapidly frozen in liquid nitrogen, and stored frozen under a blanket of argon gas at −80°C until preparation for fatty acid analysis.

Extraction of retina total lipids, separation of phospholipid classes, and fatty acid analysis. Procedures described by Marcheselli and Bazan (30) were used for homogenization of the tissue, extraction of total lipid, and separation of phospholipid classes. Samples were homogenized in chloroform/methanol (2:1, vol/vol) containing 50 mg/L butylated hydroxytoluene using a glass–glass Duall tissue homogenizer (Kontes Duall tissue grinder; Fisher Scientific, Pittsburgh, PA) (31). For completeness of extraction, the samples were sonicated for 30 min in a sonication bath prior to biphasic separation with the addition of 0.05% $CaCl_2$. The bidimensional thin-layer chromatographic procedure utilizes Silica Gel H plates containing 3% magnesium acetate as binder (Analtech Inc., Newark, DE) and solvent systems consisting of chloroform/methanol/concentrated ammonia (65:25:5, by vol) for the first dimension and chloroform/acetone/methanol/acetic acid/water (3:4:1:1:0.5, by vol) for the second dimension. Individual phospholipid fractions were visualized by spraying with a 0.005% methanolic solution of 2′,7′-dichlorofluorescein and viewing under ultraviolet light. Phospholipid classes were identified by comparison with standards (Matyrea, Inc., Pleasant Gap, PA).

Fatty acids in each phospholipid class were converted to fatty acid methyl ester (FAME) derivatives with 12% boron trifluoride in methanol (Supelco, Bellefonte, PA). Fatty acid composition of the individual phospholipids was determined by gas chromatography using a 30 m × 0.25 mm DB-225 fused silica capillary glass column (J&W Scientific, Folsom, CA). A Varian (Palo Alto, CA) 3400 gas chromatograph equipped with a flame-ionization detector and linked to a Varian 4400 integrator and a Varian autosampler were utilized. The injector and flame-ionization detector temperatures were maintained at 250 and 270°C, respectively. Helium was used as the carrier gas, with a column flow rate of 2.5 mL/s. The initial column temperature was set at 120°C. The column conditions were programmed such that the temperature increased from 120 to 170°C at a rate of 5°C per min, and from 170 to 225°C at a rate 1°C per min. FAME were identified by comparison of relative retention times with commercial FAME standards (Matyrea, Inc.) or with a secondary standard of menhaden oil obtained from NOAA Laboratory (Charleston, SC).

Statistical analysis. The data were analyzed using the general linear models procedure within SAS (32), following a randomized complete block model. Least significant difference was used for mean comparisons when significant treatment differences existed. Significant differences among means were determined at $P = 0.05$.

RESULTS

Initial body weights (mean, 1.79 kg) among the five dietary treatment groups were not significantly different. After 25 d of treatment, body weights for the four formula-fed treatment groups (mean, 10.05 kg) were heavier ($P < 0.05$) than that of the naturally-reared group (mean, 7.85 kg). No significant differences in formula consumption were observed among the four formula-fed groups.

The phosphatidylcholine (PC) fraction was responsive to dietary supplementation, with addition of DHA, AA, or BOTH resulting in increases in the added fatty acids relative to the STD (Fig. 1; Table 2). Supplementation with DHA resulted in twofold increases in percent of total fatty acids in PC–DHA with 4.88% of total fatty acids in STD compared with 10.03% in STD + DHA and 9.47% in STD + BOTH. (Fig. 1A). Other n-3 fatty acids in PC were not affected by addition of DHA or AA to the diet (Table 2). Supplementation with AA resulted in 1.3–1.4-fold increases in PC–AA, with 4.47% in STD compared with 6.19% in STD + AA and 5.70% in STD + BOTH (Fig. 1B). Significant increases in 22:4n-6 and 22:5n-6 were observed when the diet was supplemented with AA alone, but other n-6 fatty acids in PC were not affected by dietary treatment (Table 2). With respect to AA, the fatty acid profile of the naturally-reared group (SOW) was similar to that of the AA supplemented groups, i.e., AA in PC was similar in SOW, STD + AA, and STD + BOTH. The DHA in PC of the SOW was similar to that of STD + AA and STD + BOTH, but greater than the STD and

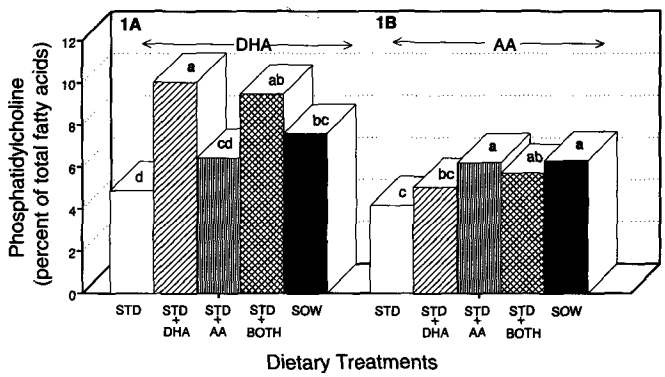


FIG. 1. DHA (1A) and AA (1B) in retina phosphatidylcholine of neonatal pigs in response to dietary treatments: STD, standard diet with fatty acid composition similar to that of some current infant formulas; STD + DHA, docosahexaenoic acid-supplemented; STD + AA, arachidonic acid-supplemented; STD + BOTH, DHA- and AA-supplemented; SOW, naturally-reared and fed sow milk. Treatment means (n = 5) not sharing a common letter are significantly different as determined by least significant difference; $P = 0.05$.

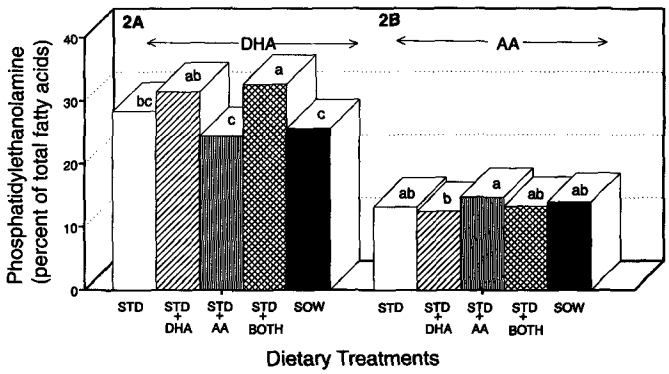


FIG. 2. DHA (2A) and AA (2B) in retina phosphatidylethanolamine of neonatal pigs in response to dietary treatments. Treatment means (n = 5) not sharing a common letter are significantly different as determined by least significant difference; $P = 0.05$. Abbreviations and diet definitions as in Figure 1.

less than the STD + DHA. No significant differences were observed among dietary treatments for any of the individual saturated fatty acids or the monoenes in PC.

For the PE fraction, supplementation with either DHA or AA alone or in combination did not result in significant increases, except for a 1.2-fold increase in DHA for STD + BOTH (32.66%) as compared with STD (28.38%) (Fig. 2; Table 3). The only other fatty acids in PE responding to dietary manipulation were 22:4n-6 and 22:5n-6, which were greater in STD + AA and SOW than in STD. Thus, under the conditions of the current experiment, retina PE was less responsive to dietary supplementation than PC.

DISCUSSION

Supplementation with DHA, AA, or BOTH clearly influenced the fatty acid profile of retina PC. Increases in accretion of these long-chain fatty acids in this phospholipid class were observed when preformed sources were provided in the diet. In contrast, the retina PE fraction, which is the phospholipid fraction containing DHA in particularly high concentrations, was less responsive to dietary supplementation of preformed C_{20-22} long chain fatty acids. The STD + BOTH treatment, but not the STD + DHA treatment, exhibited increased retina DHA in PE in response to supplementation. The changes observed for these two treatment groups compared to the STD were similar in magnitude (e.g., 31.47% for STD + DHA and 32.66% for STD + BOTH vs. 28.38% for STD), and it is possible that the small number of animals in each dietary treatment contributed to the lack of a statistically significant effect between the STD + DHA and the STD groups. Alternatively, the minimal response may be due to efficient mechanisms for acquisition and recycling of the DHA within the retina. Such mechanisms have been described by Anderson *et al.* (33) and Bazan *et al.* (34).

Innis and colleagues (35–37) have used the neonatal pig extensively to study questions similar to those addressed in the present study; however, preformed sources of AA and DHA were not used in any of these earlier studies. Hrboticky

TABLE 2
Fatty Acid Composition of Retina Phosphatidylcholine in Piglets Fed Formula Supplemented with Arachidonic Acid and Docosahexaenoic Acid[a]

	Dietary treatment[b] (% total fatty acids)						
	STD	STD + DHA	STD + AA	STD + BOTH	SOW	Pooled SEM[c]	*P*
Saturated fatty acids							
14:0	0.69	0.68	0.74	1.15	0.75	0.24	NS
16:0	32.34	34.29	32.97	35.50	34.21	1.85	NS
18:0	28.98	19.62	20.26	18.35	18.55	2.86	NS
20:0	0.30	0.09	0.10	0.10	0.12	0.11	NS
22:0	0.58	0.02	0.03	trace	0.01		
Monoenes							
16:1[d]	1.64	1.67	1.93	2.22	2.56	0.25	NS
18:1[e]	21.68	23.94	26.07	23.34	24.25	1.23	NS
20:1	0.37	0.43	0.41	0.35	0.33	0.06	NS
22:1	0.17	0.11	0.05	0.05	0.02	0.05	NS
n-6 series							
18:2n-6	1.85	2.13	1.90	1.76	2.23	0.14	NS
18:3n-6	0.19	0.18	0.21	0.19	0.27	0.02	NS
20:2n-6	0.34	0.22	0.21	0.18	0.20	0.09	NS
20:3n-6	0.33	0.38	0.33	0.35	0.34	0.03	NS
20:4n-6	4.47^{c}	$5.02^{b,c}$	6.19^{a}	$5.70^{a,b}$	6.29^{a}	0.37	0.014
22:4n-6	0.37^{b}	0.55^{b}	0.83^{a}	0.54^{b}	0.82^{a}	0.07	0.0005
22:5n-6	0.42^{b}	0.57^{b}	1.12^{a}	0.56^{b}	1.43^{a}	0.11	0.0001
n-3 series							
18:3n-3	0.09	0.06	0.04	0.05	0.03	0.02	NS
22:5n-3	0.21	0.29	0.23	0.23	0.31	0.01	NS
22:6n-3	4.88^{d}	10.03^{a}	$6.42^{c,d}$	$9.47^{a,b}$	$7.59^{b,c}$	0.80	0.0017
Summary							
SAT[f]	62.67^{a}	54.65^{b}	54.06^{b}	55.08^{b}	53.59^{b}	1.90	0.02
MUFA[f]	23.79^{b}	$26.11^{a,b}$	28.45^{a}	$25.92^{a,b}$	$27.07^{a,b}$	1.16	NS
PUFA[f]	13.10^{b}	19.24^{a}	17.49^{a}	19.00^{a}	19.33^{a}	1.06	0.003
Σ n-6[f]	7.94^{c}	$8.96^{b,c}$	$10.79^{a,b}$	$9.28^{b,c}$	11.49^{a}	0.63	0.007
Σ n-3[f]	5.16^{d}	10.27^{a}	$6.70^{c,d}$	$9.72^{a,b}$	$7.85^{b,c}$	0.79	0.0016
n-6/n-3[f]	1.73^{a}	0.89^{c}	1.64^{a}	$0.97^{b,c}$	$1.53^{a,b}$	0.20	0.025
LCn-6/LCn-3[f]	1.32^{a}	0.66^{c}	1.32^{a}	$0.77^{b,c}$	$1.21^{a,b}$	0.18	0.047
DHA/DPA[f]	$14.31^{a,b}$	19.78^{a}	5.75^{b}	20.81^{a}	5.41^{b}	3.22	0.0066

[a]Data are presented as means. Means in the same row not sharing a common, nonitalicized superscript are significantly different as determined by least significant difference; $P = 0.05$. Abbreviations as in Table 1. NS, not significant.
[b]Fatty acid composition of STD is similar to that of some current infant formulas.
[c]Pooled standard error of the mean based on n = 5, except for 22:1 and 18:3n-3.
[d]Includes 16:1n-9 and 16:1n-7.
[e]Includes 18:1n-9 and 18:1n-7.
[f]SAT, total saturated fatty acids; MUFA, total monounsaturated fatty acids; PUFA, total polyunsaturated fatty acids; Σ n-6, total n-6 fatty acids; Σ n-3, total n-3 fatty acids; n-6/n-3, total n-3 fatty acids/total n-6 fatty acids; LCn-6/LCn-3, long-chain n-6 fatty acids (C ≥ 20)/long-chain n-3 fatty acids (C ≥ 20); DPA, docosapentaenoic acid (22:5n-6).

et al. (35) showed that term-gestation piglets fed a vegetable oil-based formula with an LA/LNA ratio of 36.8 exhibited reduced DHA in retina total lipid, PC, and PE compared with piglets fed sow milk containing 0.1% DHA. Compensatory increases in 22:4n-6 and 22:5n-6 were observed in the retina of the formula-fed piglets. In subsequent studies (36,37), feeding formula with LA/LNA ratios ranging from 4 to 22 supported retina DHA accretion in PE in a manner similar to that of the SOW group with a DHA content of 0.1%; in contrast, feeding formula with an LA/LNA ratio of 30, but no preformed DHA, did not support comparable accretion of DHA in retina PE. When fish oil was used as a dietary source of DHA in a formula with 0.3% DHA and an LA/LNA ratio of 26 (36), retina PE–DHA was equivalent to that of the piglets consuming sow milk containing 0.1% DHA and having an LA/LNA ratio of 10. In the current experiment, the LA/LNA ratio of the formulas was held relatively constant at 9:1. If one uses the data of Innis and colleagues (35–37) as a frame of reference, formula containing an LA/LNA ratio of 9:1 but no added DHA should be adequate to support accretion of DHA in retina PE. This prediction is consistent with the observation that addition of preformed DHA alone did not result in significantly greater DHA in retina PE than in the piglets fed the unsupplemented formula. On the other hand, the increase in DHA in retina PE did reach statistical significance for the group fed DHA in combination with AA compared to STD. It is also noteworthy that in the SOW group, DHA in PC was significantly greater than in the STD in spite of the fact that the sow milk had an LA/LNA ratio of 22:1 and a DHA content of only 0.04% of total fatty acids.

TABLE 3
Fatty Acid Composition of Retina Phosphatidylethanolamine in Piglets Fed Formula Supplemented with Arachidonic Acid and Docosahexaenoic Acid[a]

	Dietary treatment[b] (% total fatty acids)						
	STD	STD + DHA	STD + AA	STD + BOTH	SOW	Pooled SEM[c]	*P*
Saturated fatty acids							
14:0	0.20	0.21	0.17	0.20	0.19	0.05	NS
16:0	6.97	7.46	6.34	6.24	7.86	0.79	NS
18:0	30.60	30.10	30.77	28.02	28.01	0.90	NS
20:0	0.12	0.13	0.10	0.11	0.27	0.04	NS
22:0	0.09	0.04	0.04	trace	0.04		
Monoenes							
16:1[d]	0.39	0.41	0.39	0.35	0.69	0.09	NS
18:1[e]	10.74	10.58	10.80	9.49	10.22	0.49	NS
20:1	0.19	0.16	0.22	0.20	0.15	0.02	NS
22:1	0.05	0.04	0.07	0.05	0.04	0.02	NS
n-6 series							
18:2n-6	1.30	1.13	0.95	0.91	1.04	0.10	NS
18:3n-6	0.08	0.07	0.06	0.05	0.07	0.01	NS
20:2n-6	0.18	0.17	0.20	0.25	0.19	0.02	NS
20:3n-6	0.39	0.41	0.36	0.36	0.39	0.03	NS
20:4n-6	13.17	12.46	14.70	13.24	13.91	0.53	NS
22:4n-6	$3.34^{b,c}$	2.72^{c}	4.44^{a}	$3.86^{a,b}$	4.64^{a}	0.33	0.0043
22:5n-6	3.08^{b}	1.93^{b}	5.22^{a}	3.41^{b}	6.20^{a}	0.55	0.0004
n-3 series							
18:3n-3	0.06	0.05	0.06	0.05	0.27	0.05	NS
22:5n-3	0.77	0.71	0.75	0.83	0.97	0.06	NS
22:6n-3	$28.38^{b,c}$	$31.47^{a,b}$	24.45^{c}	32.66^{a}	25.61^{c}	1.31	0.0014
Summary							
SAT	37.93	37.81	37.40	34.54	36.16	1.28	NS
MUFA	11.35	11.14	11.47	9.93	11.04	0.55	NS
PUFA	50.72	51.04	51.13	55.52	52.83	1.73	NS
Σ n-6	21.53^{b}	18.83^{b}	25.89^{a}	22.02^{b}	26.34^{a}	1.12	0.001
Σ n-3	$29.19^{b,c}$	$32.21^{a,b}$	25.24^{c}	33.51^{a}	26.49^{c}	1.34	0.0018
n-6/n-3	0.75^{b}	0.59^{c}	1.05^{a}	$0.66^{b,c}$	1.01^{a}	0.05	0.0001
LCn-6/LCn-3	0.70^{b}	0.56^{c}	1.01^{a}	$0.64^{b,c}$	0.97^{a}	0.05	0.0001
DHA/DPA	$11.44^{a,b}$	16.67^{a}	4.75^{c}	11.01^{b}	4.29^{c}	1.82	0.0009

[a]Data are presented as means. Means in the same row not sharing a common, nonitalicized superscript are significantly different as determined by least significant difference; $P = 0.05$.
[b]Fatty acid composition of the STD is similar to that of some current infant formulas.
[c]Pooled standard error of the mean based on n = 5, except for 22:1 and 18:3n-3.
[d]Includes 16:1n-9 and 16:1n-7.
[e]Includes 18:1n-9 and 18:1n-7.

In n-3 fatty acid deficiency, 22:5n-6 is increased, presumably to compensate for inadequate 22:6n-3. The ratio of DHA (22:6n-3) to docosapentaenoic acid (22:5n-6) is believed to be a more sensitive indicator of n-3 status than the relative percentages of either fatty acid (38). In the present study, the low DHA/docosapentaenoic acid ratios (4–6) observed for the STD + AA and the SOW treatments indicate that these diets were inadequate to support accretion of DHA in the retina comparable to that observed for the supplemented groups. Greater ratios (11–21) were observed in the DHA-supplemented groups for both PC and PE, but these ratios were not significantly different from those of the STD treatment group.

Because of the low DHA content of the sow milk in the current study, the naturally-reared group (SOW) should be considered a negative, rather than a positive, control with respect to DHA. However, even the very small amount of DHA in the sow milk may have played a physiological role as indicated by the fact that, in PC, DHA was greater in the SOW than in the STD. Fatty acid profiles of retina PC and PE for the SOW group were similar to those of the group supplemented with AA alone. This result can be explained by the similarities in the dietary fatty acid composition of the two groups (Table 1). In both the SOW and STD + AA groups, the DHA in the retina phospholipids was less than in the DHA-supplemented groups. These results may have implications for populations consuming large amounts of high linoleic vegetable oils and relatively little linolenic acid. Women in the United States have breast milk DHA concentrations on the low end of the spectrum compared with women in other countries (18), and it is speculated that maternal dietary supplementation of n-3 fatty acids or increased consumption of fish might be advisable.

In comparison to the retina, other tissues examined in the same study (Craig-Schmidt, M., Ping, L., Huang, M.C., Stainback, P., Mozaffar, S., and Johnson, J., unpublished data) exhibited greater fold-increases in both DHA and AA as a re-

sult of supplementation. For example, 4–6-fold increases were observed for DHA in lung and heart phospholipids, whereas the statistically significant changes in the retina were only 1–2-fold. The amount of DHA, however, is greater in the retina than in these other tissues, and because DHA is more highly conserved in the retina than in other tissues, it may be less susceptible to dietary manipulation. A lack of response to diet in retina DHA also has been observed in human infants. Makrides *et al.* (39) analyzed tissue DHA in infants undergoing postmortem examinations as a result of sudden death. Breast-fed infants had a greater proportion of DHA in their erythrocytes and brain cortex relative to those fed formula, but differences were not observed in the retina. Thus, there is some evidence that DHA in the retina may be maximized earlier than DHA in other tissues.

Carlson *et al.* (14,15) reported that preterm infants fed marine oil-supplemented formula exhibited a compromised AA status, presumably because of competition between n-6 and n-3 fatty acids. It is possible, therefore, that DHA added to formula could reduce AA in tissues. That competition exists between n-6 and n-3 fatty acids is well established (40,41), yet under conditions of the current dietary treatments, there appeared to be no significant inhibition of AA incorporation into retina phospholipid by the addition of DHA to the diet. Similarly, incorporation of DHA into retina phospholipid was not suppressed by addition of dietary AA. Retina PC was responsive to dietary supplementation, with addition of DHA, AA, or BOTH resulting in increases in these fatty acids when compared to animals fed the standard formula. Although retina PE was less responsive to dietary supplementation, addition of both DHA and AA resulted in an increase in DHA as compared to unsupplemented formula. On the basis of these results, it appears that a combination of DHA and AA in the diet can support accretion of DHA in retina phospholipids while simultaneously providing AA necessary for membrane phospholipids and eicosanoid biosynthesis.

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Relationship Between Dietary Supply of Long-Chain Fatty Acids and Membrane Composition of Long- and Very Long Chain Essential Fatty Acids in Developing Rat Photoreceptors[1]

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ABSTRACT: The present study was designed to determine if dietary supply of long-chain fatty acid (LCFA, C20:4n-6, and/or C22:6n-3), reflecting levels that might be incorporated into infant formulas, influences the fatty acid composition of the visual cell membrane. The rod outer segment (ROS) of the retina was analyzed from rats fed diets varying in the ratio of 18:2n-6 to 18:3n-3 with or without 20:4n-6 [arachidonic acid (AA)] and 22:6n-3 (docosahexaenoic acid) from birth to six weeks of age. The level of very long chain fatty acids (VLCFA, C_{24}–C_{36}) was identified using gas chromatography and gas chromatography–mass spectrometry. In the ROS, the highest relative percent of AA was attained in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of animals fed 1% AA diet, whereas feeding 0.7% docosahexaenoic acid (DHA) diet significantly increased the DHA level in PC, phosphatidylserine, and phosphatidylinositol compared to feeding diets containing AA. VLCFA of n-6 and n-3 up to C_{36} were found in PC, with the most abundant fatty acids being C_{32} and C_{34}. In PC, phosphatidylserine and PE, the n-6 tetraenoic VLCFA level was highly increased in animals fed 1% AA compared to other dietary groups. This study suggests that dietary fat containing small amounts of AA or DHA is an important factor influencing membrane fatty acid composition of the visual cell during development.

Lipids 31, 61–64 (1996).

In recent years, it has become well established that alterations in dietary fat intake result in differences in the structural lipid composition and in functions of a wide variety of membranes (1). The fatty acid composition of membrane phospholipids seems to be especially sensitive to change in the animal's intake of essential fatty acids, and the balance between the n-6 and n-3 essential fatty acids fed (2,3). A few specialized cells contain very long chain essential fatty acids (VLCFA), having chain lengths of C_{24} to C_{34} (4,5). These VLCFA are present in the brain (6,7), testes (8,9), and in the rod photoreceptors of the retina (4,10,11). The physiological role of these essential fatty acids is unknown; however, the content of VLCFA in the rod outer segment (ROS) of the retina is increased or decreased, depending on the n-6 to n-3 fatty acid balance present in the diet fat fed (4).

During development, synaptogenesis, and photoreceptor biogenesis, deficiency of 22:6n-3 results in profound biochemical changes in membrane composition and membrane function of the ROS. In the preterm human infant, feeding 22:6n-3 seems to improve the delayed recovery of the dark-adapted electroretinogram and visual acuity (12–14). This suggests that n-3 fatty acids exert a functional role in the development of normal visual function that is dependent on the utilization of n-6 and n-3 fatty acids derived from the diet. Both 20:4n-6 and 22:6n-3 give rise to longer chain homologues in the ROS of the retina (4). This raises questions of what is the impact of dietary 20:4n-6 and 22:6n-3 content on the level of VLCFA homologues and the functional contributions of these fatty acids. Therefore, the present study was designed to determine if dietary supply of 20:4n-6 and 22:6n-3, reflecting levels that might be incorporated into infant formula, influences the fatty acid content of the major phospholipid components of the ROS in the retina.

MATERIALS AND METHODS

Animals and diets. Sprague-Dawley rats, obtained from the University of Alberta vivarium (Edmonton, Alberta, Canada), were housed individually, unless breeding. During breeding, three females and one male were housed together for a two-week "mating" period. Females were then moved to individual cages. Room conditions were maintained at 21°C with 12-h light and 12-h dark cycle. Water and food were supplied *ad libitum*. Experimental diets were fed to dams following delivery of the rat pups. All litters were culled to twelve animals within 24 h following parturition. Pups were sacrificed by decapitation in the light at three and six weeks of age. Animals

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; GC–MS, gas chromatography–mass spectrometry; LCFA, long-chain fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine, ROS, rod outer segment; TLC, thin-layer chromatography; VLCFA, very long chain fatty acids.

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at three weeks of age received only maternal milk with no access to the chow fed the dam. Animals sacrificed at six weeks of age had been weaned at three weeks of age and were fed the same diet received by the dam.

Five semipurified experimental diets containing 20% fat (w/w) and varying in fat composition were fed (Table 1). Diet fats were formulated based on the fat composition of an existing infant formula providing an n-6/n-3 ratio of 7.3:1 (SMA; Wyeth-Ayerst Laboratories, Radnor, PA). Four experimental diets were formulated by the addition of various triglycerides to alter the composition of this basic fat formulation (Table 1). The arachidonic acid (AA) and docosahexaenoic acid (DHA) triglycerides utilized were obtained from single-cell oils (Martek Corporation, Columbia, MD).

Isolation of retina and ROS. All isolations were performed at 4°C. Retinas were isolated (15). Six retinas were pooled for separation of the ROS. ROS were isolated by discontinuous sucrose gradient centrifugation (16). Aliquots of ROS were evaluated by microscopy and polyacrylamide gel electrophoresis (17). In the gel, opsin with a molecular weight of 35000 daltons was present in ROS membranes. The purified ROS fraction contained only minor subcellular contamination by retinal debris.

Lipid analysis. Total lipids were extracted from the ROS (18). Phospholipids were separated from neutral lipids using silica G-plates (20 × 20 cm) and developed in a solvent system composed of petroleum ether/diethyl ether/acetic acid (80:20:1, by vol) and then eluted three times with 10 mL chloroform/methanol (1:1, vol/vol) containing 1 ppm ethoxyquin. Phospholipids were dried under nitrogen and redissolved in 0.2 mL hexane. Phospholipids were separated by spotting on hexane prewashed silica-gel H-plates (20 × 20 cm) developed in chloroform/methanol/2-propanol/0.25% (wt/vol)KCl/triethylamine (30:9:25:6:18, by vol) (19) for 1-1/2 h followed by chloroform/methanol/1-propanol/0.25% (wt/vol) KCl/triethylamine (30:9:25:6:18, by vol) for 1-1/2 h. With these two systems, phosphatidylcholine (PC) was separated from sphingomyelin. Thin-layer chromatography (TLC) plates were dried at room temperature for five minutes and visualized with 0.01% (wt/vol) Anilino Naphthalene sulfonic acid in water. Each phospholipid was recovered, and fatty acid methyl esters were prepared using BF_3/methanol reagent (20).

Analysis of fatty acid methyl esters. For separation of long-chain fatty acid (LCFA) and VLCFA, a Hewlett-Packard 5890 GLC (Palo Alto, CA) equipped with a Vista DS 654 data system (Varian Instruments, Georgetown, Ontario, Canada) was used. Chromatography utilized a BP × 70 column (25 m × 0.22 mm i.d.). The carrier gas was helium, at a flow rate of 2.2 mL/min and a split ratio of 20:1. The initial oven temperature was started at 110°C, increased to 320°C at a rate of 3°C/min and then held for five minutes. The injector temperature was 250°C. With these conditions, VLCFA up to 36 carbons in chain length were separated. Identification of the longer chain length fatty acids was based, in part, on retention times. Identification of the degree of unsaturation of the fatty acid was based on gas chromatography–mass spectrometry (GC–MS) analysis and prior separation of fatty acid methyl esters on the basis of a number of double bonds by argentation TLC.

Fatty acid methyl esters were injected into the BP × 70 column and analyzed by an electron ionization mass selective detector. The injector temperature was 250°C, and the GC–MS transfer line temperature was 300°C. The identification of VLCFA was performed using a Hewlett-Packard 5970 mass selective detector with a Hewlett-Packard comp 216 data system. The mass spectra of very-long-chain tetra, penta, and hexaenoic methyl esters from PC contained patterns similar to those published earlier (4). Argentation TLC was used to resolve fatty acid methyl esters on the basis of degree of unsaturation as described earlier (4).

Statistical methods. The effect of diet on fatty acid composition of phospholipid in ROS was assessed by analysis of variance procedures. Significant effects of treatment were defined utilizing Duncan's multiple range test (21). All data is expressed as mean ± standard error of the mean.

TABLE 1
Fatty Acid Composition of Diets Fed[a]

Fatty acid (wt% of fat)	n-6/n-3 7.3:1	n-6/n-3 4:1	1% AA	0.7% DHA	AA + DHA
12:0	9.6	7.0	7.3	10.2	7.9
14:0	5.8	5.1	4.8	6.2	5.7
16:0	12.7	14.6	15.7	14.7	15.9
18:0	7.7	8.1	9.3	7.3	8.9
18:1(n-7 + n-9)	40.7	40.4	38.9	38.3	38.8
18:2n-6	15.8	17.6	16.2	16.0	16.8
18:3n-3	2.2	4.3	2.3	2.3	2.3
20:4n-6	—	—	1.2	—	1.2
20:5n-3	—	—	—	—	—
22:6n-3	—	—	—	0.8	0.7
n-6/n-3	7.3	4.1	7.1	6.9	7.5

[a]n-6/n-3, the ratio of 18:2n-6 to 18:3n-3; AA, arachidonic acid; DHA, docosahexaenoic acid.

RESULTS AND DISCUSSION

Diet treatment did not alter the weight gain of the animals in this experiment. In previous experiments, feeding n-3 fatty acids compared to low or deficient n-3 fatty acid intake in an n-6 to n-3 fatty acid unbalanced diet results in changes in the levels of n-3 fatty acids present in brain and retina. In the present study, however, a physiological level of intake of all fatty acids (22) was provided (Table 1). Inclusion of relatively small amounts of 20:4n-6 and 22:6n-3 in the postweaning diet increased the level of 20:4n-6 in PC and phosphatidylethanolamine (PE) (Fig. 1). It is also noteworthy that feeding 22:6n-3 is also associated with increases in the 20:4n-6 content of both phospholipids in the ROS compared to n-6/n-3 of 7:1 and 4:1 diet. This close association between the incorporation of 20:4n-6 and 22:6n-3 has been reported for brain phospholipids (23) and is highly suggestive that a diet induced increase in 22:6n-3 content in PC, and phosphatidylser-

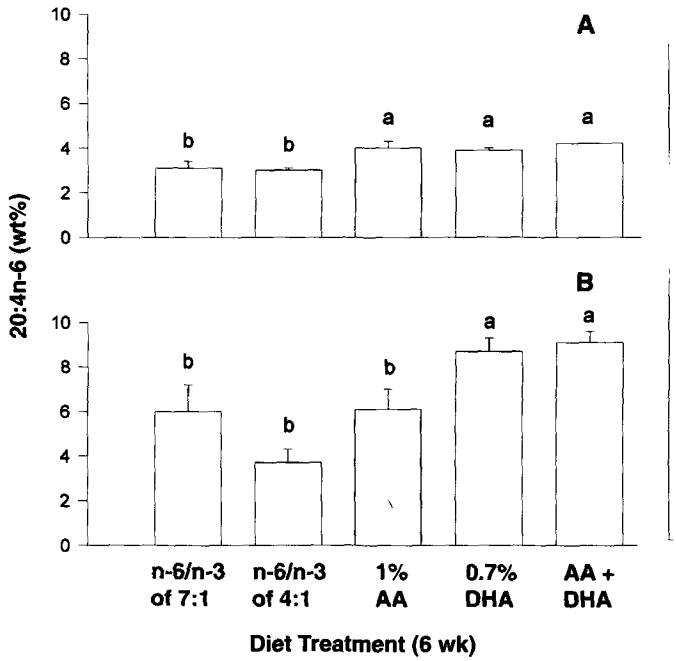


FIG. 1. Effect of diet fat on the content of 20:4n-6 in (A) phosphatidylcholine and (B) phosphatidylethanolamine. Values are mean ± SEM (wt%) with n = 3–5 for each group. Six retinas were pooled for each n. Significant effects of diet treatment were defined, utilizing Duncan's multiple range test at the level of $P < 0.05$ for a vs. b. Retinas were collected in the regular laboratory light; AA, arachidonic acid; DHA, docosahexaenoic acid.

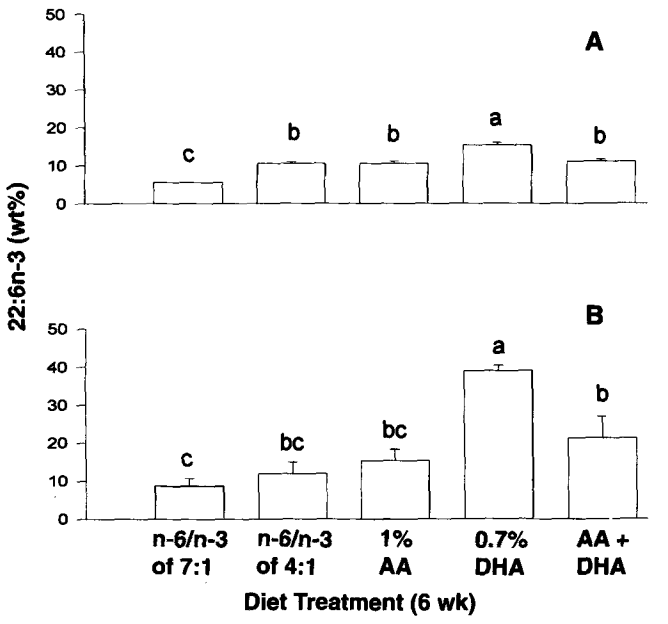


FIG. 2. Effect of diet fat on the content of 22:6n-3 in (A) phosphatidylcholine and (B) phosphatidylserine in the rod outer segment. Values are mean ± SEM (wt%) with n = 3–5 for each group. Six retinas were pooled for each n. Significant effects of diet treatments were defined, utilizing Duncan's multiple range test at the level of $P < 0.05$ for a vs. b vs. c. Retinas were collected in the regular laboratory light; see Figure 1 for abbreviations.

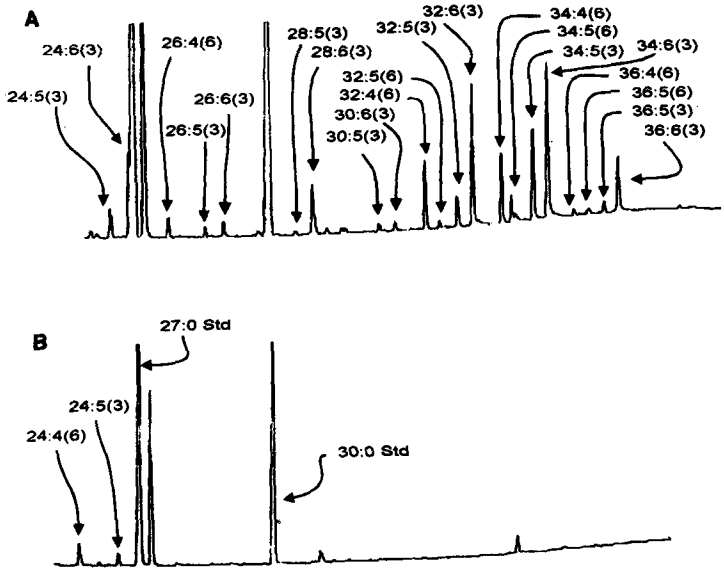


FIG. 3. Content of very long chain fatty acids of rod outer segment in phosphatidylcholine (A) and phosphatidylethanolamine (B). Identification of the each fatty acid peak was verified by gas chromatography–mass spectrometry. Phosphatidylcholine (A) contains very long chain fatty acids with up to 36 carbons in chain length, whereas phosphatidylethanolamine (B) contains up to 24 carbons. Std, standard.

ine (PS) in the ROS (Fig. 2) results in the synthesis of highly diunsaturated phospholipid species containing 20:4n-6 and 22:6n-3.

In the postweaning ROS, feeding of 22:6n-3 either alone or with 20:4n-6 increased phospholipid content of 22:6n-3 (Fig. 2). In PC, increasing the 18:3n-3 content of the diet resulted in an increase in the content of 22:6n-3. In PS, feeding 22:6n-3 increased the content of 22:6n-3 from 2.5- to 4-fold (Fig. 2).

In the ROS, only PC contains significant levels of VLCFA, with up to 36 carbons in chain length and having 4, 5, and 6 double bonds (Fig. 3). These observations are consistent with reports for bovine retina (24), human brain, and rat brain (6,25). Feeding 20:4n-6 in the diet was effective in increasing the level of n-6 tetraenoic VLCFA present in PC (Fig. 4), PE, and PS (data not shown), with concomitant reductions in n-3 VLCFA, which was vice versa with feeding 22:6n-3. The content of n-6 pentaenoic VLCFA was small, and the difference was not significant except C32:5n-6 in PC.

The present study provides experimental evidence indicating that small dietary amounts of 20:4n-6 and 22:6n-3 are important factors influencing membrane fatty acid composition of the visual cell during development. The data in this presentation has focused on the early postweaning period. However, our evidence indicates that analogous effects of diet also occur during the preweaning period. These effects of diet include significant alterations in LCFA and VLCFA in phospholipids as well as a reciprocal replacement of n-6 tetranoic VLCFA by n-3 pentaenoic VLCFA. These VLCFA of C_{24} to

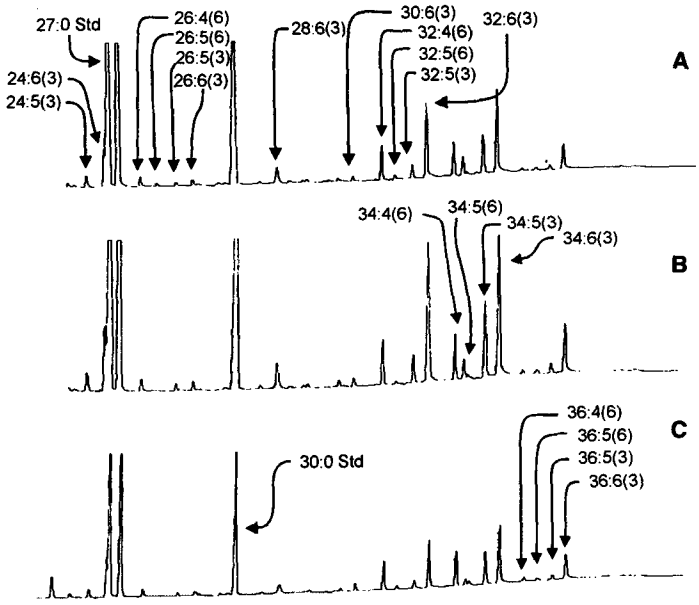


FIG. 4. Effect of feeding 20:4n-6 on the content of very long chain fatty acids in phosphatidylcholine in rod outer segment. Identification of each fatty acid peak was verified by gas chromatography–mass spectrometry. (A), The ratio of n-6/n-3 = 7.3:1; (B), (A) + 0.7% 22:6n-3 (DHA); (C), (A) + 1% 20:4n-6 (AA). See Figures 1 and 3 for abbreviations.

C_{36} carbon chain length are not of direct dietary origin and must be synthesized from shorter chain essential fatty acid precursors present in the diet fed. The functional roles for very long-chain n-6 and n-3 fatty acids in the ROS remain to be determined.

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The Effect of Docosahexaenoic Acid on the Electroretinogram of the Guinea Pig[1]

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ABSTRACT: Docosahexaenoic acid (DHA), an n-3 polyunsaturated fatty acid, is found in consistently high concentrations in the retinae of mammals, yet its role in vision remains unclear. In this study, a mammalian model of variable retinal DHA concentration has been developed, such that the retinal phospholipids of guinea pigs contained between 2.5 and 30.8% DHA. Visual function was assessed using full-field flash electroretinography, over a range of exposure levels spanning six log units. Trend analysis indicated that retinal function was altered by the tissue DHA level, and was described by a second-order polynomial "inverted U-shaped" function. The results suggested that although some amount of DHA is essential for normal retinal function, increases in the DHA level past an optimal amount, found to be 19%, provided diminishing returns. In this study, manipulation of the retinal DHA level accounted for 21–35% of the electroretinographic variability.

Lipids 31, 65–70 (1996).

The most ubiquitous of the retinal n-3 polyunsaturated fatty acids (PUFA) is docosahexaenoic acid (DHA), which is contained in up to 60% of all structural phospholipid molecules, within the membranes of the photoreceptor outer segments (1). The prevalence and constancy of n-3 PUFA, particularly DHA (2,3), are clues that it plays an important role within the retina. Despite this, the function of DHA is still unclear.

Attempts to deplete mammals, such as rat and monkey, of retinal n-3 fatty acids by dietary manipulation, in an endeavor to elucidate the nature of DHA function, have had limited success (4,5). In these studies, complete depletion of DHA was not possible due to transplacental transport and other conservation mechanisms (6). Nevertheless, there is evidence to suggest that even DHA depletion to a level 60% or below control affects retinal function. In particular, n-3 deficiency has been shown to give rise to electroretinographic deficits (1,7–9), losses in visual acuity (1,10), and learning defects (11). In addition, n-3 deficiency has been shown to directly affect the function of the visual photopigment, rhodopsin (12), and a model for the role of DHA in modulation of rhodopsin function has been proposed (13).

The aim of the present study was to use an animal model of variable retinal DHA content (14) (ranging from negligible to very high) in order to assess the effect of this fatty acid on visual function.

MATERIALS AND METHODS

All experimental and surgical procedures were approved by the Animal Experimentation Ethics Committees of the Melbourne and Deakin universities and were conducted in accordance with the "Australian code of practice for the care and use of animals for scientific purposes."

Sixteen commercial diet-fed female Duncan-Hartley guinea pigs (in groups of four) were weaned onto a guinea pig commercial diet (LC—laboratory chow), or one of three semisynthetic diets based on a premixture described by Leat *et al.* (15). Various oils, providing a range of n-3 intakes, were added to this premixture to provide the sole source of lipid for each diet. The diet designed to result in depletion of retinal n-3 PUFA was the safflower oil (SO)-based diet, which utilized safflower seed oil as the only source of lipid. SO contains negligible amounts of n-3 and high amounts of n-6 fatty acids (n-6/n-3 = 72.0). Canola oil (CO) (n-6/n-3 = 2.5) was added to a second diet. The CO diet, like the LC diet (n-6/n-3 = 8.9) served as an n-3-sufficient diet. The third oil-supplemented diet contained a mixture of fish oil (FO), providing very high levels of n-3 fatty acids, particularly the longer chain n-3 PUFA, eicosapentaenoic acid, and DHA, plus CO and SO (n-6/n-3 = 2.5). The FO was added to the premixture in the form of cellulose-coated microcapsules to prevent oxidation (Dry n-3™ powder; Dano Chemo A/S, Norway). These oils were added in the ratio of fish/canola/safflower (25:45:30), and in all three synthetic diets this lipid comprised 100 g per 1 kg of diet mixture. Commercial chow was the fourth group, added as a standard reference, and all pellets originated from a single batch to ensure constancy throughout the experiment. All diets were supplemented with 10 g fresh carrots daily and water containing ascorbic acid (400 mg/L) *ad libitum*. The light environment was cycled (12 h light, 12 h dark) in the animal housing room and tempera-

[1]Based on a presentation at the AOCS Annual Meeting & Expo in San Antonio, Texas, May 7–11, 1995.

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Abbreviations: 22:5n-6, Docosapentaenoic acid; CO, canola oil; DHA, docosahexaenoic acid; ERG, electroretinogram; FO, fish oil; LC, laboratory chow (commercial chow); PUFA, polyunsaturated fatty acids; SO, safflower oil.

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ture was constant at 21°C. Fatty acid compositions of the four diets measured as described by Weisinger *et al.* (14) are given in Table 1.

Female guinea pigs were reared on these diets to four months of age, at which time they were joined with LC-fed males. Offspring (second generation guinea pigs) were weaned onto their respective diets at approximately 14 d. Second generation females were joined with LC-fed males. The offspring of this union (third generation, 4–6 litters giving n = 12 per diet group) were assessed at six to nine weeks of age, there being no significant difference between the ages of animals from different groups (mean age ± SD: SO, 7.0 ± 1.4; LC, 6.8 ± 0.9; CO, 6.5 ± 0.5; FO, 6.5 ± 0.9).

Following deep anesthesia (0.035 mL Ketamine and 0.025 mL Xyalzine/kg body weight) and mydriasis (Tropicamide 0.5%), full-field white flash electroretinograms (ERG) were recorded by placement of chlorided silver wire electrodes into the vitreous (active) and inferior cul de sac (inactive). Light flashes were delived *via* a fiber optic, positioned 6 mm from the cornea, at six light exposure levels, spanning the operational limits of both rod and cone receptor systems; the dimmest exposure was 1 lux.s. Exposure was raised in one log unit increments with the brightest flash being 10^5 lux.s. For each exposure, eight repeats were averaged to give the final waveform. The duration of each flash was 5 ms at the nominal exposure. The interstimulus interval was 15 s (between flashes). All animals were dark-adapted overnight (18 h) prior to the recordings.

Immediately following electroretinography, animals were sacrificed and their retinae removed under normal room light conditions. Lipids were extracted from the retinal tissue using chloroform/methanol, 2:1 (containing butylated hydroxytoluene antioxidant), and the phospholipids were separated from the neutral lipids by thin-layer chromatography (16). The methyl esters of the phospholipid fatty acids were formed by a saponification step using KOH followed by transesterification in BF_3 in methanol (16), and the fatty acid methyl esters were separated by capillary gas–liquid chromatography (DANI 6500 HR; ICI International, Melbourne, Australia) using a 50 m × 0.32 mm (i.d.) fused silica bonded phase column (BPX70; SGE, Melbourne, Australia). The column oven was programmed at 125°C for 3 min to 220°C at 8°C/min with helium as carrier gas at a flow rate of 43 cm/s. Fatty acids were identified by comparison with standard mixtures of fatty acid methyl esters, and the results were calculated using response factors derived from chromatographing standards of known composition (Nu-Chek-Prep, Elysian, MN).

The ERG is conventionally recognized by the measurement of two major changes in retinal polarity following light stimulation, the initial "negative" a-wave, and the ensuing "positive" b-wave. The a-wave strongly represents the activity within the photoreceptors, and the b-wave represents that of post-receptoral processes. The peak-to-peak amplitude is equivalent to the b-wave amplitude, taking into account the initial a-wave negativity and, hence, is a superior measure of the positive-polarity forces generated in the post-receptoral networks. Both peak-to-peak and a-wave amplitudes were measured, and the relationship between these electroretinographic waveforms, and the retinal DHA content was determined using trend analysis (17). The degree of correlation between these parameters was estimated using the Spearman statistic.

TABLE 1
Fatty Acid (FA) Composition of Diets (expressed as g FA/100 g total FA)

	Diet group			
Fatty acid	Fish oil[a]	Canola oil	Laboratory chow	Safflower oil
14:0	2.11	0.38	0.49	0.41
16:0	8.23	5.15	16.21	7.30
16:1	2.34	0.28	0.23	0.14
18:0	3.08	2.70	3.51	2.63
18:1	34.18	58.93	31.07	15.04
18:2n-6	31.44	20.55	39.49	71.99
18:3n-3	4.06	8.19	4.46	1.01
20:0	0.78	1.01	0.52	0.32
20:1	1.31	1.47	0.54	0.08
18:4n-3	0.75	—	—	—
20:4n-6	0.30	—	—	—
20:5n-3	4.28	—	—	—
22:1	0.60	0.38	0.39	—
22:5n-3	0.58	—	—	—
22:6n-3	2.80	—	—	—
24:0	0.20	—	0.37	—
24:1	0.15	—	—	—
Total n-6	31.74	20.55	39.49	71.99
Total n-3	12.47	8.19	4.46	1.01
n-6/n-3	2.54	2.51	8.85	71.27

[a]The oil-supplemented diets (fish, canola, and safflower oils) were composed of 10% lipid (by weight); the laboratory chow diet contained 4.9% lipid.

RESULTS AND DISCUSSION

The results given in Table 2 indicate that the retinal fatty acid profile can be manipulated by dietary means by six to nine weeks of age in third generation animals. The retinal DHA content was found to be directly proportional to the dietary supply of precursory n-3 fatty acids (Fig. 1), consistent with, and extending, the earlier work of Leat *et al.* (15) on this species.

Both the n-6/n-3 ratio and total n-3 (by weight) were considered in the analysis of the diet–tissue DHA relationship. The n-6/n-3 ratio is considered a better index of dietary n-3 supply than the absolute quantity of added n-3 fatty acids. This is because n-6 and n-3 fatty acid families compete for the enzymes of desaturation (18). Therefore, a high total n-3 in the diet is less effective in increasing tissue n-3 PUFA (such as DHA) levels when the n-6 fatty acid content of the diet is also high. However, the usefulness of this index is reduced where the metabolic desaturation pathway is bypassed by direct supply of longer-chain PUFA. The FO diet, for example, supplies both eicosapentaenoic acid and DHA (in addition to α-linolenic acid), but contains an n-6/n-3 ratio nearly identical to that of the CO diet (2.54 and 2.51, respectively). As is shown in Table 2, animals in the FO group had greater levels of n-3 fatty acids in their retinae than those of the

TABLE 2
Fatty Acid (FA) Composition (g FA/100 g phospholipid FA ± SEM) of Retinal Phospholipids from Guinea Pigs Fed Diets Containing Different Levels of n-3

	Diet group			
Fatty acid[a]	Fish oil (n = 11)	Canola oil (n = 12)	Laboratory chow (n = 13)	Safflower oil (n = 13)
16:0	17.60 ± 0.09	17.90 ± 0.19	17.60 ± 0.12	17.60 ± 0.19
18:0	21.30 ± 0.36	21.00 ± 0.10	21.70 ± 0.28	21.70 ± 0.24
18:1	11.00 ± 0.08	11.67 ± 0.12[b]	11.17 ± 0.13	9.29 ± 0.12[b]
18:2n-6	1.33 ± 0.07	1.63 ± 0.08[b]	1.30 ± 0.06	2.62 ± 0.11[b]
20:3n-6	0.71 ± 0.02	0.76 ± 0.02	0.59 ± 0.01	0.85 ± 0.02
20:4n-6	7.26 ± 0.11[b]	9.23 ± 0.10	9.55 ± 0.15	9.84 ± 0.10
20:5n-3	0.48 ± 0.03	—	—	—
22:4n-6	1.07 ± 0.03[c]	2.53 ± 0.04[c]	3.05 ± 0.05	4.53 ± 0.05[c]
22:5n-6	**0.53 ± 0.04[c]**	**5.23 ± 0.09[c]**	**14.08 ± 0.18**	**23.32 ± 0.49[c]**
22:5n-3	1.78 ± 0.05[c]	1.34 ± 0.03[c]	0.75 ± 0.26	0.22 ± 0.01[c]
22:6n-3	**30.82 ± 0.31[c]**	**20.98 ± 0.15[c]**	**12.36 ± 0.35**	**2.50 ± 0.23[c]**
24:4n-6	0.39 ± 0.02[c]	1.60 ± 0.03[c]	1.93 ± 0.06	2.66 ± 0.09[c]
24:5n-6	—	0.10 ± 0.01[c]	0.21 ± 0.01	0.22 ± 0.01
24:5n-3	0.84 ± 0.06[c]	0.59 ± 0.02[c]	0.24 ± 0.01	0.02 ± 0.01[c]
24:6n-3	0.45 ± 0.02[c]	0.20 ± 0.00[c]	—	—

[a]DHA (22:6n-3) and 22:5n-6 values are presented in bold text.
[b,c]Significantly different from laboratory chow group, [b]$P < 0.01$, [c]$P < 0.001$.

canola group. Indeed, the retinal DHA content in the FO group was about 30% higher than the canola group.

In summary, these diet groups enabled the assessment of animals varying dramatically in terms of retinal DHA proportion, ranging from negligible amounts in the retinae of SO-supplemented animals to over ten times that proportion in the retinae of FO-supplemented animals. In agreement with the literature, the changes in retinal DHA concentration were inversely proportional with changes to the levels of docosapentaenoic acid (22:5n-6), and it appears that, biochemically, 22:5n-6 and DHA are interchangeable within the phospholipids of the retina. Together, DHA and 22:5n-6 comprise approximately 26–31% of the total retinal phopholipid fatty acid. The diets were very consistent in their effects on the fatty acid profile, with the standard deviation between animals in a given group rarely higher than 5%. For DHA content, the average standard deviation of the diet groups was 3.44%.

Other features of the retinal fatty acid profiles were that the diet with the highest n-6/n-3 (SO) had the highest levels of all n-6 PUFA ($P < 0.05$). The total n-6 level in the SO group was 44.04 and the n-3 level was 2.74%. As the proportion of the n-3 PUFA increased in the diet relative to the n-6 levels, the levels of n-3 PUFA increased in the retinal phospholipids. Thus, the total n-6 and total n-3 PUFA in commercial chow were 30.71 and 13.39%, respectively; in the CO diet the values were 21.08 and 23.15%, respectively; and for the FO group the values were 11.29 and 34.37%, respectively. Therefore, between 44 and 47% of the retinal phospholipid fatty acids were PUFA. The proportion of saturated fatty acids (16:0 and 18:0) and oleic acid (18:1) remained fairly constant between diet groups (see Table 2).

Figure 2 illustrates typical ERG recorded from four animals (representative of the four dietary groups) at the 10 lux.s exposure. Both a-wave and b-wave components are evident at this illumination level.

Table 3 gives the outcomes for the trend analysis between ERG amplitude and retinal DHA content. It shows the F-ratios for the zero order (constant), first order (linear), and second order polynomial when incorporated into the trend, as well as their applicability to the data. In modelling this data, a higher order polynomial was only included when the F-ratio was significantly ($P < 0.05$) greater than the preceeding lower order function.

Although there were significant differences in many of the other retinal PUFA (beside DHA and 22:5n-6), for example, a reduction in the arachidonic acid level in FO-fed animals, which may have contributed to the observed electroretinographic changes as a function of diet, these were not significantly correlated.

As is evident in Table 3, the second order regression provided the best fit to the data giving the (highest) F-ratio and r^2 values. We did not consider higher than second order polynomials as these were not statistically significant, and there is no postulated physiological basis for such a relationship. The results indicate that DHA serves a vital role in the normal function of the retina and that retinal DHA content forms a complex (nonlinear) relationship with the electroretinographic output. An inverted U-shaped trend (Scheme 1) was evident for the relationship between DHA content and the a-wave amplitude ($P < 0.02$) when the flash exposure was between 2.0 and 5.0 log lux.s and for the peak-to-peak amplitude ($P < 0.02$) at all intensities except 2.0 and 4.0 log lux.s. The most likely cause for the absence of any trend in the a-wave at the dimmest exposures is the low signal-to-noise ratio found at these levels. The implicit times of the a-wave and b-wave were measured, and no significant difference was found between any of the diet groups.

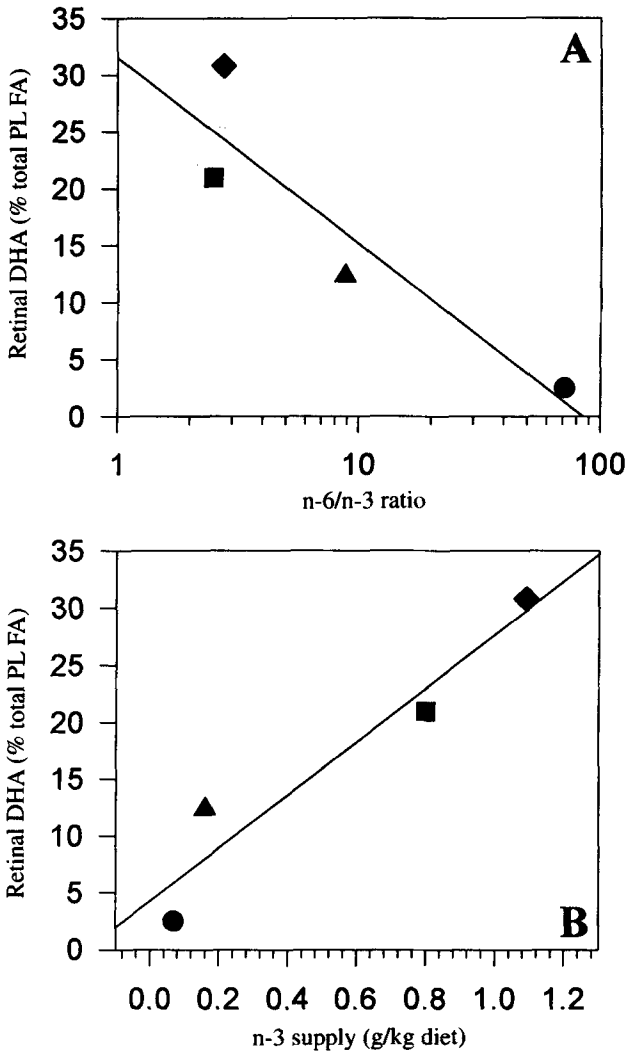


FIG. 1. A. Retinal docosahexaenoic acid (DHA) level as a function of dietary n-6/n-3 supply ratio. Fish oil (diamond), canola oil (square), laboratory chow (triangle), and sunflower oil (circle) diet groups, and the corresponding average (error bars smaller than symbol) retinal DHA levels. B. Retinal DHA as a function of total n-3 fatty acids included in the diet (g/kg diet). The lines are best fitting linear regressions. PL, phospholipids; FA, fatty acid.

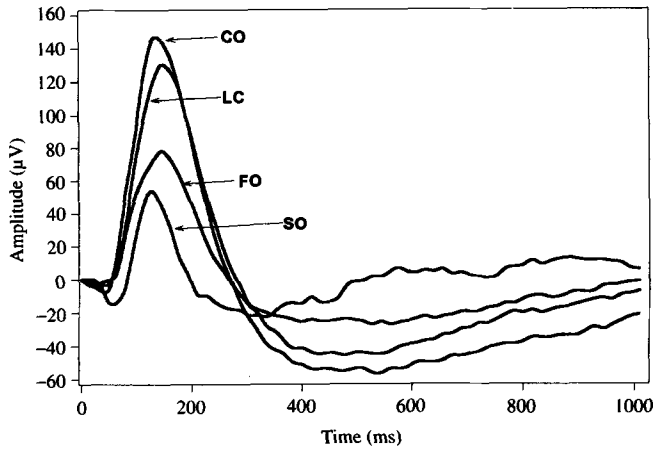


FIG. 2. Electroretinograms from a single animal taken at the 10 lux.s exposure level, representative of data obtained from the four diet groups (SO, safflower oil; CO, canola oil; FO, fish oil; and LC, laboratory chow).

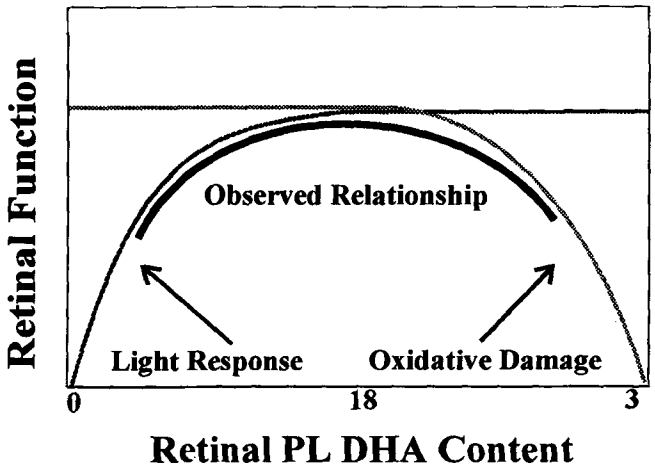
The tissue–DHA level predicted to give optimal retinal function was determined by applying differential calculus to the second order polynomials to find the maximum for each light exposure level. The results indicate that a value of between 15–26% gives the optimal retinal DHA content (Table 4). The averages of 18.01 ± 1.89 (a-wave) and 20.74 ± 3.47 (peak-to-peak) suggest that a content of about 19% (corresponding to an n-3 supply of 0.64 g/kg of diet; n-6/n-3 ratio of 5.7, see Fig. 1) would give the best electroretinographic outcome. This value corresponds closely to those levels found in the LC- and CO-supplemented groups. Notably, the r^2 values obtained from this analysis indicate that between 21–35% of the variation in electroretinographic responses can be explained by a change in the retinal DHA content (see Table 3).

The mechanism by which alteration in retinal DHA content affects visual function is not known. It has been suggested that a reduced DHA content may directly impair the process of phototransduction (12), or perhaps synaptic transmission to the post-receptoral network (19). Notably, each rhodopsin molecule is surrounded by approximately 60 phospholipid molecules, most of which contain DHA as the major constituent (20). Hence, depletion of DHA could be expected to alter photoreceptor function. Such losses are observed despite the absence of structural damage to the retinal tissue (21) and an apparent increase in rhodopsin content within the outer segment disks (12). Interestingly, Watanabe *et al.* (22) found a decrease in the number of large phagosomes in the retinal pigment epithelium, but no structural change to the sensory retina. Landis *et al.* (23) observed an absence of photoreceptor disk shedding in animals fed a fat-free diet. These observations indicate that a reduction in retinal DHA content produces subtle inefficiencies in phototransduction and disk recycling mechanisms rather than morphological abnormalities. On the other hand, a high DHA content leads to oxidative stress. Receptor numbers become reduced, outer-seg-

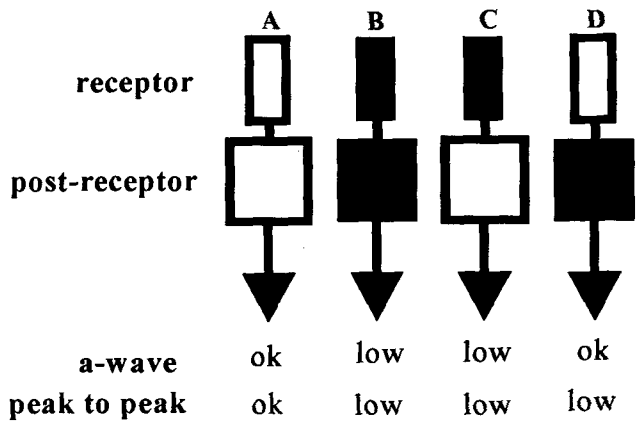
TABLE 3
Analysis of Electroretinographic Peak Amplitude[a] as a Function of Retinal Docosahexaenoic Acid Content for the Polynomial Fitted (as described in the text)

Exposure	a-Wave			Peak-to-peak		
(log lux.s)	r^2	F	*P*	r^2	F	*P*
5.0	**0.21**	**5.77**	**0.020**	**0.27**	**8.29**	**0.010**
4.0	**0.26**	**7.46**	**0.010**	0.12	2.96	0.090
3.0	**0.29**	**8.86**	**0.000**	**0.22**	**5.99**	**0.020**
2.0	**0.30**	**9.03**	**0.000**	0.07	1.69	0.200
1.0	0.24	3.02	0.100	**0.35**	**11.54**	**0.000**
0.0	0.10	1.26	0.270	**0.29**	**8.88**	**0.000**

[a]Listed are the square of the coefficient, r^2, F-ratio (F), and statistical significance (*P*) values for all second order polynomial regressions. Significant correlations ($P < 0.05$) are indicated by bold text.



SCHEME 1



SCHEME 2

ments are smaller, and post-receptoral physiology is disrupted by oxidative or phototoxic insult (24). This insult is likely to have occurred during rearing in room lighting conditions, as the experiments of Penn and Anderson (24) show that it can occur at normal room light levels (300 lux). Furthermore, our study determined no significant difference between the optimal DHA concentrations for high and low flash exposures (Table 4). If the primary effect of photic/oxidative insult was occurring during the flash exposure used in the experiment, a lower optimum DHA content would have been expected under conditions of high light exposure (Table 4). This is contrary to what was actually observed.

A model to describe the inverted U-shaped effect of DHA content on retinal physiology is shown in Scheme 1. This model proposes two opposing lipid-related factors influencing retinal function. The first factor is one that influences phototransductional light capture. The other involves oxidative damage. Furthermore, a scheme has been proposed to describe the electroretinographic findings in terms of receptoral (rhodopsin modulation) or post-receptoral (oxidative) involvement in Scheme 2. When the DHA content is low, reduced peak-to-peak and a-wave amplitudes are a result of photoreceptor dysfunction due to inefficiencies in light capture (Scheme 2, C). As discussed earlier in this chapter, this dysfunction probably arises from less efficient rhodopsin function (such as reduced "quantal catch") associated with the changes in membrane characteristics. When the content of DHA in the retinal phospholipid approaches 19%, retinal function peaks (Scheme 2, A). However, when the DHA content is raised above this optimal level, reduced amplitudes arise as a result of oxidative damage either at the photoreceptor level (Scheme 2, C) or both receptorally and post-receptorally (Scheme 2, B). This model is supported by our experimental data given in this paper and by the work of Penn and Anderson (24), who reported marked structural damage to the retinae of animals raised on high n-3 fatty acid diets in normal and in bright light conditions.

TABLE 4
Retinal Docosahexaenoic Acid (DHA) Content That Is Predicted by Trends to Give the Greatest Electroretinographic Peak-to-Peak at 10^2 lux.s and a-Wave

Flash exposure (log lux.s)	Optimal retinal DHA (% of total phospholipid fatty acids) a-Wave	Peak-to-peak
5.0	20.21	21.20
4.0	19.90	26.18
3.0	18.61	20.68
2.0	17.54	N/A[a]
1.0	16.03	18.60
0.0	15.79	17.04
Mean ± SD	18.01 ± 1.89	20.74 ± 3.47

[a]No sensible correlation was obtained for peak-to-peak at 10^2 lux.s.

The present study refutes the notion that a high content of DHA is necessary for optimal visual function as optimal retinal function occurred in the presence of moderate levels of DHA (19%). However, the retinal fatty acid profile of the guinea pig, in particular the proportion of DHA, appears different from that of other mammals. Whereas DHA comprises up to 60% of the retinal fatty acids in control diet-fed rats (25) and monkeys (19), the retinae of guinea pigs in this study contained half of this fraction (the FO group). However, we note that the high DHA levels were taken from rod outer segments rather than whole retinas as in the present study. The "control" groups, CO and LC, had DHA levels much lower than that of the FO group (see Table 2). This finding is in agreement with those of Leat *et al.* (15), who found similarly "low" DHA levels in chow-fed guinea pigs.

The fact that the guinea pig is the only mammal studied that can be completely depleted of retinal DHA suggests that it metabolizes n-3 fatty acids differently. Indeed, optimal retinal function is achieved in the guinea pig where the retinal DHA level is even lower than that of rats and monkeys raised on n-3–deficient diets. This apparently lowered demand for high tissue DHA levels is consistent with the lack of conservation of DHA in guinea pigs.

Two final points need to be raised. Our findings indicate that DHA plays a key role in the retinal function of the guinea

pig and that optimal retinal function is achieved in the presence of some 22:5n-6. It is also important to recognize that our study was limited to a single age group. Others have shown that there is an age dependency of the effect of n-3 deficiency on ERG function, with older animals showing little or no ERG anomalies (9).

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Artificial Rearing of Infant Rats on Milk Formula Deficient in n-3 Essential Fatty Acids: A Rapid Method for the Production of Experimental n-3 Deficiency[1]

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ABSTRACT: Research into the function of docosahexaenoic acid (DHA; 22:6n-3), the predominant polyunsaturated fatty acid (PUFA) in the central nervous system (CNS), is often hindered by the difficulty in obtaining dramatic experimental decreases in DHA in the brain and retina of laboratory rats. In this study, the artificial rearing procedure, whereby infant rats are removed from their mothers, gastrostomized, and fed synthetic formula, was used in an attempt to produce rapid changes in CNS levels of DHA. Female rats were raised, from day 4–5 of life, on one of two formulas—one containing the essential fatty acids of both the n-6 and n-3 series in proportions approximately equal to those of rat milk, and the other containing high levels of 18:2n-6 but very little n-3 fatty acid. At weaning, both groups were given AIN-76A diets modified so that the PUFA content resembled that of the preweaning formula. At eight weeks of age, the n-3-deficient group exhibited decreases of more than 50% in total DHA content in the brain, accompanied by increases in arachidonic acid (AA) (20:4n-6) and, especially, docosapentaenoic acid (22:5n-6). Other artificially-reared rats were mated and their offspring were also maintained on the respective diets. In spite of the fact that they had been reared artificially, the rats mated successfully and reared litters with no obvious abnormalities. At both ten days of age and again at eight weeks, offspring of the n-3-deficient mothers exhibited decreases of more than 90% in total DHA content. Again, the long-chain n-6 PUFA increased proportionately so that total PUFA levels in the brain were not lower. As these differences are greater than those commonly reported, even after 2–3 generations of normal dietary deprivation in rodents, this procedure may be an important tool in the study of the effects of n-3 deficiency on neural development and, subsequently, of the function of DHA in nervous tissue.

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A basic principle of nutritional research is that the determination of the essentiality of a nutrient requires the manipulation of tissue levels of that nutrient and the evaluation of appropriate functional endpoints. Specifically, this strategy requires that the following criteria be met: (i) the removal of the nutrient from the food source; (ii) the maintenance of the subject for an appropriate duration so that tissue levels decline to subnormal levels; and (iii) the evaluation of changes in structure or function of that tissue. In the case of the n-3 essential fatty acids (EFA), however, this approach has proven to be difficult. Although n-3 EFA can be removed from the food source either by providing, as the sole source of fat, oils such as safflower oil or through the use of specific purified fatty acids, the second criterion (the maintenance of the subject until tissue levels of n-3 EFA have been reduced sufficiently) has been difficult to meet. The member of the n-3 family which is of major interest, docosahexaenoic acid (DHA; 22:6n-3), is the predominant polyunsaturated fatty acid (PUFA) in the central nervous system (CNS) of mammals (1–4), and DHA in the brain of many species accrues mostly from mother's milk during the suckling period, when much of the brain is undergoing the most rapid stage of development. Furthermore, DHA is selectively retained by the brain, making depletion of brain stores difficult after weaning, when brain development is almost complete and the CNS has accumulated most of the adult concentration of 22:6n-3. The depletion of DHA, if begun after weaning, can take most of the lifetime of the animal, and major decreases often require a second or even third generation to be maintained on the diet. For example, in the rat, the usual procedure has been to place postweanling females on n-3-deficient diets throughout their lives and then evaluate their offspring, and although this technique has led to drastic reductions in 22:6n-3 in the brain in the second generation according to some reports (5,6), many studies have reported more modest decreases (7–9). Continuing the dietary n-3 deprivation for a third generation resulted in further decreases (10–13), but the time and resources required to carry out such studies may be prohibitive for many researchers.

One alternative approach has been to use animals, such as the nonhuman primate or the young piglet, which can be removed from the nursing mother shortly after birth and fed a

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Abbreviations: AA, arachidonic acid; AR, artificial rearing; CNS, central nervous system; DHA, docosahexaenoic acid: EFA, essential fatty acids; PUFA, polyunsaturated fatty acid.

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synthetic formula devoid of n-3 EFA throughout much of the brain development period. This method has been used successfully in a number of laboratories, producing major declines in CNS levels of DHA during early life in both monkeys (14–16) and piglets (17,18). Unfortunately, although the feeding of infant formula to monkeys and pigs provides a powerful model for the experimental production of n-3 deficiency, it has two practical limitations. Because of the considerable length of time required for these species to reach adulthood, and the space requirements for their housing and maintenance, use of these animals can require resources beyond the reach of many laboratories. Also, the common tests which would be used to evaluate functional changes in the CNS are less developed, and often more unwieldy, in these species than in common laboratory rodents.

The laboratory rat, on the other hand, is the subject of choice for most researchers. It is relatively easy and inexpensive to acquire and maintain, it reaches adulthood relatively quickly, and more is known about the behavioral capabilities of the rat than about any other nonhuman species. Furthermore, the relatively few reports of effects of n-3 deficiency suggest that DHA is required for normal retinal (12,19) and brain function (2,4,20). What is needed to facilitate further research into the nature of these effects is a method of producing rapid and dramatic declines in DHA in the rat brain.

Fortunately, there is a well-developed technique for raising infant rats on synthetic formula. The artificial rearing (AR) method for rats was developed a quarter century ago (21,22), and with subsequent advances in the development of appropriate rat-milk substitutes (23,24), it enables the researcher to make relatively precise manipulations of specific nutrients during a critical period of development (for review, see Ref. 25). AR has provided researchers with a powerful tool in such fields as early nutrition (24) and the effects of prenatal ethanol exposure (26). This paper describes the first attempt to use AR of infant rats to produce rapid decreases of DHA in the brain.

MATERIALS AND METHODS

Subjects. The first generation of rats were offspring of timed-pregnant Sprague-Dawley rats purchased from Taconic Farms (Germantown, NY). Upon arrival in our animal facility, the dams were individually housed in plastic cages on Bed o' Cobs bedding (Anderson Company, Maumee, OH) and maintained under a 12-h dark/light cycle at 21 ± 1°C. All offspring were housed, after weaning, in same-sex groups of up to five per cage.

Chow diets. Upon arrival, the pregnant dams were maintained on NIH-31 lab chow (Zeigler Brothers, Gardners, PA). After weaning, AR offspring were maintained first on modified AIN-76A powdered diets (BioServe, Frenchtown, NJ) and were eventually transferred to modified AIN-76A pelleted diets (Dyets, Inc., Bethlehem, PA). In both cases, the diet was modified in two ways: (i) the protein was comprised of vitamin-free casein, as we had determined that this protein source contained less fat than did casein; and (ii) the fat content was 5% by weight, and comprised of saturated fats in the form of distilled monoglycerides (Myverol 18-06 K; Eastman Chemicals, Inc., Kingsport, TN) and unsaturated fats in the form of ethyl esters of purified linoleate and linolenate (Nu-Chek-Prep, Elysian, MN), and docosahexaenoate (a kind gift from Professor Yasushi Tamura, Chiba University, Chiba City, Japan). The fatty acyl composition of the diets is shown in Table 1. In the control group, 12% of the fat was in the form of linoleic acid while 1% each was provided by linolenic acid and DHA. In the experimental group, there were almost no n-3 fatty acids, while 26% of the fat was in the form of linoleic acid.

Rat-milk substitute. The neonatal pups used in the AR treatment were fed a rat-milk substitute adapted from a protocol used elsewhere (J. Edmond, personal communication), and the composition is shown in Table 2. The rat-milk substitute consisted of a milk-protein substitute (provided as a gift by Professor John Edmond; UCLA, Los Angeles, CA) to which was added amino acids, carbohydrate, fat, vitamins, and minerals to approximate the composition of rat milk. During the first two days of AR, the diet was modified somewhat in that half the protein was in the form of soy protein (Supro Plus 675; Protein Technologies International, St. Louis, MO). This change was made after pilot work in our laboratory showed that partial substitution of soy protein for casein reduced mortality due to gastrointestinal bloat in the first few days of rearing. The fat content of the diet was composed of short- and medium-chain saturated fatty acids provided by medium-chain triglyceride (MCT) oil (Mead Johnson, Evansville, IN) and pure ethyl esters of the EFA linoleic and

TABLE 1
Fatty Acyl Composition of n-3-Adequate and n-3-Deficient Chow Diets[a]

	Area percent	
Fatty acid	n-3-Adequate diet	n-3-Deficient diet
Nonessential		
16:0	11.0	9.4
18:0	72.3	61.4
18:1n-9/n-12[b]	0.3	0.5
Total NEFA	85.2	72.6
n-6 PUFA		
18:2	12.0	26.3
n-3 PUFA		
18:3	1.1	0.06
22:5	0.04	ND[c]
22:6	0.8	ND
Total n-3 PUFA	2.0	0.06
Total (%)	99.2	98.9
n-3/n-6 ratio	0.163	0.002

[a]NEFA, nonessential fatty acids; PUFA, polyunsaturated fatty acids.
[b]18:1n-9 also contains n-12 isomer.
[c]ND indicates not detected.

TABLE 2
Composition of Rat-Milk Substitute

Ingredient	g/kg Diet
Protein	
Milk protein extract (SW8707)[a]	88.84
Whey protein[b]	3.21
Custom amino acid mixture[c]	0.85
Carbohydrate	
Polycose[d]	28.6
Custom fat mixture	
Custom fat mixture	123.2
Vitamins	
ICN vitamin mixture	3.52
Custom supplemental vitamin mixture[e]	0.48
Minerals	
Custom calcium mixture[f]	7.92
Noncalcium mineral mixture[g]	5.30
$CuSO_4$ solution (0.03 g/mL)	0.85
$ZnSO_4$ solution (0.38 g/mL)	0.26
Other Ingredients	
Carnitine (0.1 g/mL)	0.35
Creatine solution (0.01 g/mL)	6.16
Ethanolamine	0.03

[a]Gift from Dr. John Edmond (UCLA, Los Angeles, CA).
[b]Alacen Extra 865 (New Zealand Milk Products, Santa Rosa, CA).
[c]Arginine (512 g/kg), glycine (310 g/kg), taurine (156 g/kg), picolinic acid (22 g/kg).
[d]From Ross Laboratories (Columbus, OH).
[e]Riboflavin (16.7 g/kg), niacin (26 g/kg), pyridoxal (13.9 g/kg), inositol (929.4 g/kg).
[f]Calcium phosphate (720 g/kg), calcium chloride (205 g/kg), calcium hydroxide (75 g/kg).
[g]KH_2PO_4 (812 g/kg), $MgSO_4$ (152 g/kg), $FeSO_4$ (4 g/kg), KI (0.29 g/kg), NaF (0.246 g/kg), $AlSO_4$ (0.156 g/kg), $MnSO_4$ (0.042g/kg).

TABLE 3
Fatty Acyl Composition of n-3-Adequate and n-3-Deficient Liquid Diets[a]

	mg Fatty acid/mL diet	
Fatty acid	n-3-Adequate diet	n-3-Deficient diet
Nonessential		
8:0	48.4	36.3
10:0	22.5	18.8
16:0	0.5	0.5
18:0	0.2	0.3
18:1n-9	0.4	0.5
Total NEFA	72.5	56.9
n-6 PUFA		
18:2	12.3	38.6
n-3 PUFA		
18:3	1.2	0.03
20:5	0.01	ND[b]
22:6	0.9	ND
Total n-3 PUFA	2.2	0.03
Total (mg/mL)	87.0	95.5
n-3/n-6 Ratio	0.176	0.001

[a]Abbreviations as in Table 1.
[b]ND indicates not detected.

α-linolenic acid, and the long-chain PUFA DHA. The fatty acid composition of the two diets is shown in Table 3.

Procedure. On day 4 or 5 after birth, pups were lightly anesthetized with isofluorane and implanted with gastrostomy tubes as described elsewhere (27,28). Briefly, six-inch-long Intramedic tubing (Clay Adams, Parsippany, NJ) (PE 10, 0.61 mm o.d.) was lubricated with MCT oil, inserted into the mouth, passed down the esophagus and out the lateral wall of the stomach. The pups were then housed on bedding in small plastic containers floating in heated water (36 ± 1°C). The milk substitute was delivered for 10 min every hour *via* PE 50 tubes attached to syringes mounted on infusion pumps (Harvard Apparatus, South Natick, MA). The total amount of food delivered each day was equal to 33% of the body weight of the pup for the first 3–4 d, and then gradually increased over the next week until it was 40% of body weight each day. Approximately equal numbers of pups from each litter were assigned to each experimental group. Pups were weaned on days 18–19 onto solid diets corresponding in PUFA content to the liquid diets. Some AR rats were killed at approximately eight weeks of age for lipid analysis, and others were allowed to reach sexual maturity and were mated beginning at 16 wk of age. Their offspring were allowed to suckle with their mothers and were removed and housed in same-sex groups on day 25 ± 1 d. They were then fed the diets fed to their mothers. Some pups were killed by decapitation on day 10 for fatty acid analysis, and others were killed by CO_2 exposure at eight weeks of age. In both cases, no more than one animal from each litter was used for biochemical analysis. All animal procedures were carried out in accordance with the guidelines set by the Animal Welfare Act and were done with the approval of the NIAAA Animal Care and Use Committee.

Biochemical analysis. Lipids were extracted from brain homogenates with chloroform and methanol by the method of Bligh and Dyer (29). Fatty acid methyl esters of total lipids were prepared with 14% (wt/vol) boron trifluoride and methanol according to the procedure of Morrison and Smith (30), and analyzed by a gas chromatograph (Hewlett-Packard 5890; Hewlett-Packard, Palo Alto, CA) equipped with a flame-ionization detector and a 30 m × 0.25 mm (i.d.) capillary column (J&W DB-FFAP; J&W, Folsom, CA). The conditions were as follows: 130°C initial temperature, no hold time, increase 4°C/min to 175°C, 1°C/min to 210°C, 30°C/min to 240°C, hold for about 15 min. Individual fatty acids were quantified by adding known amounts of the fatty acid 23:0 to the original homogenate before extraction, and the fatty acids were identified by comparison with known standards.

Statistical analysis. Data were analyzed as μg of fatty acid per gram of tissue by *t*-tests using the StatView statistical analysis package for the Macintosh computer. Significance level was set at $P < 0.01$.

RESULTS

The forebrain and cerebellar fatty acid composition of adult rats reared artificially from days 4–5 of life are shown in

TABLE 4
Effects of Feeding n-3-Deficient Diet Beginning on Day Five of Life in Adult (8 wk) Rat Forebrain Fatty Acyl Composition[a]

	μg/g Forebrain (wet weight)		
Fatty acid	n-3-Adequate diet	n-3-Deficient diet	*P*-value
Nonessential			
14:0	163 ± 3	107 ± 8	0.0245
16:0	4611 ± 27	4931 ± 42	0.0217
16:1n-7	102 ± 2	103 ± 1	0.8316
18:0	4546 ± 25	4889 ± 34	0.0070
18:1n-9	3618 ± 15	3699 ± 36	0.3789
18:1n7	841 ± 7	930 ± 10	0.0110
Total NEFA	15174 ± 75	16046 ± 136	0.0365
n-6 PUFA			
18:2	81 ± 1	86 ± 1	0.2027
20:3	106 ± 2	82 ± 2	0.0022
20:4	2267 ± 15	2642 ± 11	<0.0001
22:4	680 ± 3	917 ± 6	<0.0001
22:5	150 ± 3	1964 ± 34	<0.0001
Total n-6 PUFA	3301 ± 20	5715 ± 42	<0.0001
n-3 PUFA			
22:5	36 ± 1	9 ± 0	<0.0001
22:6	3259 ± 22	1666 ± 21	<0.0001
Total n-3 PUFA	3298 ± 21	1674 ± 21	<0.0001
Total (ug/g)	21773 ± 112	23436 ± 181	
n-3/n-6 Ratio	1.00	0.29	<0.0001

[a]Data are expressed as the mean ± SEM for n = 5 animals each. Abbreviations as in Table 1.

TABLE 5
Effects of Feeding n-3-Deficient Diet Beginning on Day Five of Life in Adult (8 wk) Rat Cerebellum Fatty Acyl Composition[a]

	μg/g Cerebellum (wet weight)		
Fatty acid	n-3-Adequate diet	n-3-Deficient diet	*P*-value
Nonessential			
14:0	180 ± 17	231 ± 9	0.2796
16:0	4148 ± 78	4945 ± 128	0.0449
16:1n-7	177 ± 17	219 ± 9	0.3627
18:0	4509 ± 73	4892 ± 169	0.3796
18:1n-9	4190 ± 69	4267 ± 185	0.8656
18:1n-7	1192 ± 24	1343 ± 51	0.2628
Total NEFA	16780 ± 289	18608 ± 708	0.3163
n-6 PUFA			
18:2	131 ± 3	137 ± 5	0.6770
20:3	129 ± 3	122 ± 5	0.5682
20:4	1810 ± 34	2292 ± 86	0.0474
22:4	567 ± 13	993 ± 39	0.0016
22:5	102 ± 3	2408 ± 85	<0.0001
Total n-6 PUFA	2786 ± 55	6015 ± 208	0.0002
n-3 PUFA			
22:5	49 ± 1	12 ± 1	<0.0001
22:6	3687 ± 63	1550 ± 41	<0.0001
Total n-3 PUFA	3736 ± 64	1563 ± 41	<0.0001
Total (ug/g)	23302 ± 407	26185 ± 945	
n-3/n-6 ratio	1.34	0.26	<0.0001

[a]Data are expressed as the mean ± SEM for n = 5 animals each. Abbreviations as in Table 1.

Tables 4 and 5. At eight weeks of age, the levels of n-3 PUFA, particularly 22:6n-3, in both the forebrain and cerebellum declined considerably in the group reared on n-3-deficient rat-milk substitute neonatally and weaned onto deficient chow, and this decrease was accompanied by an increase in the amount of n-6 PUFA, particularly 22:5n-6 and, to a lesser extent, 20:4n-6 and 22:4n-6. The percentage decline of 22:6n-3 in the cerebellum was slightly greater than that in the forebrain (58% vs. 49%).

Most AR rats mated successfully and gave birth to live litters (15/16 in the adequate group vs. 12/15 in the deficient group). In several cases in both groups, all pups within a litter died within a few days of birth (five litters in the adequate group vs. three litters in the deficient group). Under visual examination, surviving pups appeared to be normal in both groups. Because the precise day of copulation was not known in these animals, it is not known if sexual responsiveness or gestation length differed between groups.

The fatty acid composition of brain regions of ten-day-old offspring of AR rats is shown in Tables 6 and 7. The n-3 PUFA declined by over 90% in both the forebrain and cerebellum of the n-3-deficient pups, and again, this drop was accompanied by increases in n-6 fatty acids. Similar declines of 22:6n-3 and associated increases in 22:5n-6 and other n-3 PUFA were also seen in the forebrain and cerebellum of eight-week-old offspring (Tables 8 and 9).

DISCUSSION

In this study, rats which had been fed n-3-deficient diets from day 4–5 of life and throughout development and adulthood exhibited losses of approximately 50% in n-3 fatty acids—particularly 22:6n-3—in the brain. Furthermore, their offspring exhibited losses of greater than 90% in 22:6n-3 in the brain both during the neonatal period and in early adulthood. These losses were offset by increases in long-chain n-6 fatty acids—primarily 22:5n-6, but also 20:4n-6 and 22:4n-6—so that the total PUFA content of the brain was maintained as described previously (31,32).

Although a recent study reported dramatic decreases in 22:6n-3 in the brains of offspring of guinea pigs fed n-3-deficient diets from weaning (33), the decreases found in the current study are of a much greater magnitude than those reported in rats or mice in most studies which rely on feeding of n-3-deficient laboratory chow beginning at weaning in the first generation. Although some early reports (5,6) described reductions of as much as 90–95% in phospholipid 22:6n-3 in the brain in the second generation, later studies generally have reported more modest decreases of approximately 50–75% (7–9). Even when the dietary n-3 deprivation was continued for a third generation, the resulting 22:6n-3 levels were still between one-half to one-quarter of those of n-3-adequate controls (10–13),

TABLE 6
Fatty Acyl Composition of Forebrain in Ten-Day-Old Rat Pups of Artificially-Reared Dams[a]

	μg/g Forebrain (wet weight)		
Fatty acid	n-3-Adequate diet	n-3-Deficient diet	*P*-value
Nonessential			
14:0	201 ± 5	214 ± 4	0.3034
16:0	3675 ± 55	3878 ± 92	0.3829
16:1n-7	168 ± 3	177 ± 3	0.3780
18:0	2085 ± 26	2151 ± 58	0.6212
18:1n-9	1354 ± 14	1330 ± 32	0.7435
18:1n-7	371 ± 4	380 ± 9	0.6357
Total NEFA	7918 ± 103	8202 ± 197	0.5470
n-6 PUFA			
18:2	105 ± 3	98 ± 1	0.2781
18:3	5 ± 0	6 ± 0	0.2708
20:3	79 ± 3	48 ± 0	0.0012
20:4	1719 ± 20	2044 ± 50	0.0237
22:4	337 ± 4	484 ± 10	0.0004
22:5	232 ± 11	1712 ± 51	<0.0001
Total n-6 PUFA	2495 ± 38	4406 ± 108	0.0002
n-3 PUFA			
22:5	27 ± 0	3 ± 0	<0.0001
22:6	1766 ± 22	130 ± 4	<0.0001
Total n-3 PUFA	1799 ± 22	134 ± 4	<0.0001
Total (ug/g)	12212 ± 161	12742 ± 309	
n-3/n-6 Ratio	0.72	0.03	<0.0001

[a]Data are expressed as the mean ± SEM for n = 4 animals each. Abbreviations as in Table 1.

TABLE 7
Fatty Acyl Composition of Cerebellum in Ten-Day-Old Rat Pups of Artificially-Reared Dams[a]

	μg/g Cerebellum (wet weight)		
Fatty acid	n-3-Adequate diet	n-3-Deficient diet	*P*-value
Nonessential			
14:0	179 ± 12	145 ± 13	0.3527
16:0	2102 ± 141	1720 ± 136	0.3696
16:1n-7	107 ± 8	87 ± 6	0.3715
18:0	1232 ± 80	1041 ± 84	0.4353
18:1n-9	1102 ± 91	892 ± 72	0.4177
18:1n-7	305 ± 24	253 ± 19	0.4512
Total NEFA	5156 ± 359	4249 ± 334	0.3963
n-6 PUFA			
18:2	80 ± 6	61 ± 5	0.3203
18:3	3 ± 0.2	2 ± 0.2	0.5031
20:3	51 ± 4	28 ± 2	0.0665
20:4	922 ± 62	894 ± 76	0.8843
22:4	227 ± 15	286 ± 27	0.3148
22:5	121 ± 7	684 ± 59	0.0013
Total n-6 PUFA	1415 ± 92	1965 ± 169	0.1546
n-3 PUFA			
22:5	23 ± 2	2 ± 0.4	0.0087
22:6	975 ± 61	80 ± 8	0.0016
Total n-3 PUFA	1002 ± 63	82 ± 9	0.0017
Total (ug/g)	7573 ± 514	6296 ± 511	
n-3/n-6 Ratio	0.710	0.041	<0.0001

[a]Data are expressed as the mean ± SEM for n = 4 and n = 3 animals each, respectively. Abbreviations as in Table 1.

considerably higher than those of the second generation in this study.

When rats were given n-3-deficient diets beginning during the neonatal period, the losses of 22:6n-3 in the cerebellum exceeded those in the forebrain, and both structures exhibited similar losses when deficiency occurred throughout all stages of development. This may have been due to the fact that, in the rat, the cerebellum undergoes most of its development—and subsequent accrual of n-3 fatty acids—during the postnatal period while the forebrain has already undergone much of its development at the time of birth (34).

It has been reported in a number of studies (10,35,36) that n-3 deficiency is associated with increased frequency of reproductive failure, and n-3 fatty acids are reported to be necessary for the normal uterine development in the female rat (37). Some of the AR rats in the present study exhibited some difficulty in caring for their pups in the first few days after birth, but the frequency of this finding was approximately similar in both deficient and adequate dams. It is more likely that this problem was caused by the early removal of the rats from their own mothers and/or the AR experience itself. Future studies will address this issue in a quantitative fashion by evaluating maternal behavior in deficient and in adequate dams.

Earlier, the point was made that the determination of the essential nature of a nutrient required the removal of the nutrient from the food source, the maintenance of the subject for an appropriate duration so that tissue levels decline to subnormal levels, and the evaluation of any change in structure or function of that tissue. Because much of the DHA in the brain has accrued by weaning in most mammalian species, dietary n-3 deprivation beginning after this period may be relatively inefficient due to the time required to substantially reduce DHA levels in the brain. In species such as the primate or pig, feeding of n-3-deficient formulae can eliminate dietary n-3 EFA during brain development, but practical considerations make the use of these species difficult for many researchers. The rat, on the other hand, is an ideal experimental subject due to its relatively brief generation time, the relative ease with which it can be housed and maintained, and the vast amount of information available regarding its behavioral repertoire. In this study, through the use of the AR method, we have shown that the rat may also be an ideal subject for studies requiring dietary deprivation during much of the period of brain development. Future work in our laboratory will further address the precise nature of these biochemical changes at the level of the individual phospholipid classes and molecular species, and the question of functional changes in

TABLE 8
Fatty Acyl Composition of Forebrain in Adult Offspring of Artificially-Reared Dams[a]

Fatty acid	µg/g Forebrain (wet weight) n-3-Adequate diet	n-3-Deficient diet	*P*-value
Nonessential			
14:0	216 ± 7	206 ± 3	0.5972
16:0	5366 ± 50	5152 ± 28	0.1364
16:1n-7	163 ± 5	151 ± 2	0.3433
18:0	5043 ± 41	4787 ± 33	0.6612
18:1n-9	4129 ± 48	3622 ± 15	0.0019
18:1n-7	930 ± 10	920 ± 5	0.6957
Total NEFA	17179 ± 174	16196 ± 59	0.0435
n-6 PUFA			
18:2	75 ± 1	75 ± 1	0.9887
20:3	104 ± 3	73 ± 1	0.0010
20:4	2553 ± 13	2728 ± 13	0.0028
22:4	771 ± 6	1011 ± 5	<0.0001
22:5	208 ± 5	3209 ± 13	<0.0001
Total n-6 PUFA	3726 ± 17	7114 ± 30	<0.0001
n-3 PUFA			
22:5	29 ± 1	ND[b]	<0.0001
22:6	3734 ± 36	307 ± 5	<0.0001
Total n-3 PUFA	3763 ± 36	307 ± 5	<0.0001
Total (ug/g)	24668 ± 222	23617 ± 84	
n-3/n-6 Ratio	1.01	0.04	<0.0001

[a]Data are expressed as the mean ± SEM for n = 4 animals each. Abbreviations as in Table 1.
[b]ND indicates not detected.

TABLE 9
Fatty Acyl Composition of Cerebellum in Adult Offspring of Artificially-Reared Dams[a]

Fatty acid	µg/g Cerebellum (wet weight) n-3-Adequate diet	n-3-Deficient diet	*P*-value
Nonessential			
14:0	130 ± 13	147 ± 3	0.5867
16:0	3941 ± 100	4224 ± 69	0.3270
16:1n-7	128 ± 13	135 ± 3	0.8265
18:0	3989 ± 72	3941 ± 62	0.8272
18:1n-9	3722 ± 92	3369 ± 58	0.1848
18:1n-7	1002 ± 23	1044 ± 15	0.5223
Total NEFA	14644 ± 336	14679 ± 234	0.9698
n-6 PUFA			
18:2	97 ± 2	104 ± 2	0.2736
20:3	100 ± 3	87 ± 1	0.1204
20:4	1636 ± 19	1968 ± 35	0.0058
22:4	498 ± 6	905 ± 16	<0.0001
22:5	115 ± 3	2785 ± 42	<0.0001
Total n-6 PUFA	2474 ± 29	5889 ± 95	<0.0001
n-3 PUFA			
22:5	30 ± 1	6 ± 0.3	<0.0001
22:6	3317 ± 67	314 ± 2	<0.0001
Total n-3 PUFA	3347 ± 67	320 ± 2	<0.0001
Total (ug/g)	20465 ± 420	20889 ± 327	
n-3/n-6 Ratio	1.35	0.06	<0.0001

[a]Data are expressed as the mean ± SEM for n = 4 animals each. Abbreviations as in Table 1.

the CNS resulting from severe DHA deficiency in neural tissue.

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Arachidonic Acid Supply and Metabolism in Human Infants Born at Full Term[1]

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ABSTRACT: Infants need arachidonic acid (AA; C20:4n-6) for eicosanoid synthesis and deposition in growing tissues, including brain. Human milk supplies preformed AA in amounts considered to meet accretion in membrane-rich tissues, but vegetable oil-based infant formulas do not contain AA. We studied two groups of ten healthy infants, each fed human milk or formula, and analyzed plasma lipid composition. Percentage contributions of AA to plasma phospholipids were stable over two months after birth in breast-fed infants, but infants fed formula developed significantly ($P < 0.05$) lower levels at the ages of two weeks (formula 6.9% vs. breast 9.4%, w/w), one month (6.2 vs. 9.1%), and two months (5.7 vs. 8.4%). In a second trial, we randomized infants to receive (from birth to age four months) formula without or with both AA and docosahexaenoic acid (DHA; C22:6n-3) at levels typical for mature human milk. Infants fed conventional formula showed a continuous decrease of phospholipid AA over time, whereas feeding of formula supplemented with AA and DHA led to significantly higher AA levels, similar to those in breast-fed infants (two months: supplemented 9.6% vs. unsupplemented 7.1%; four months: 8.7 vs. 6.6%). In order to estimate infantile capacity for endogenous synthesis of AA, we fed four term neonates with newly diagnosed phenylketonuria (mean age 18 d) a formula with all fat contributed by corn oil, which has a higher natural ^{13}C-enrichment than European human milk or formula. Analysis of ^{13}C-enrichment in plasma fatty acids over four days allowed us to estimate infantile AA synthesis. We found an increased ^{13}C-value in plasma AA of all infants, which indicates that term neonates can synthesize AA. However, with a simplified isotope balance equation, we estimate that endogenous synthesis contributed only about 23% of total plasma arachidonic acid by day four. We conclude that full-term infants fed formula may require a dietary supply of some preformed AA if the biochemical status of breast-fed infants is to be achieved.
Lipids 31, 79–83 (1996).

Docosahexaenoic acid (DHA) is considered a conditionally essential nutrient for preterm infants, because its dietary supply was shown to be related to visual function in the first months of life and to cognitive development at a corrected age of one year (1–3). It is also generally agreed that DHA supply for preterm infants should not interfere with arachidonic acid (AA) status, as we and others found availability of AA related to growth (4–6). Therefore, it has been recommended in Europe that formula for low-birthweight infants should contain preformed elongation/desaturation products of both linoleic acid (LA) and α-linolenic acid (ALA) (7,8).

More recent results have raised the question whether healthy infants born at full term may also benefit from a dietary supply of preformed long-chain polyunsaturated fatty acids (LC-PUFA). Human milk (HM) provides considerable amounts of LC-PUFA (9,10), and formula based on vegetable oils does not (11,12). This difference in dietary intake is reflected by reduced DHA contents of structural brain lipids in formula-fed infants, as was shown by studies on tissue samples of deceased infants (13,14). Moreover, some studies using visual-evoked potential measurements found a lower visual acuity during the first months of life in formula-fed than in breast-fed babies (15,16), which has been correlated to DHA status (15), although other investigators did not find differences in visual function assessed with simple behavioral methods (17). However, a recent study with randomized feeding of formula without or with preformed DHA found a significant benefit of DHA on visual acuity as determined by evoked potentials (18). Less information is available on the metabolism and biological functions of AA in term babies. Some studies on formula-fed infants found lower AA values in plasma and red blood cell lipids than in breast-fed infants (19,20), while others did not (21,22). To shed some further light on this question, we studied AA metabolism and the effect of diet in full-term infants.

EXPERIMENTAL PROCEDURES

Subjects. In a first study, we followed 20 healthy infants born at full term, of which 10 were breast-fed and 10 fed a conventional infant formula based on cow's milk protein and vegetable fat (Pre-Aptamil; Milupa, Friedrichsdorf, Germany) from birth to age eight weeks (23). The formula used contained LA and ALA in amounts (13.2 and 1.0% w/w, respectively) comparable to those typical for mature HM in Europe (10), but no detectable AA and DHA. Blood samples for fatty

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Abbreviations: AA, arachidonic acid (C20:4n-6); ALA, α-linolenic acid (C18:3n-3); DGLA, dihomo-γ-linolenic acid (C20:3n-6); DHA, docosahexaenoic acid (C22:6n-3); GLA, γ-linolenic acid (C18:3n-6); HM, human milk; LA, linoleic acid (C18:2n-6); LC-PUFA, long-chain polyunsaturated fatty acids; ‰, per thousand.

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acid analyses were obtained at the ages of two weeks, one month, and two months.

In a second study, 22 apparently healthy, full-term, and appropriate for gestational age infants fed formula were randomly assigned (on day 5 of life) to be fed either with the same LC-PUFA–free formula used in the previous study (Pre-Aptamil; n = 10) or a formula with equal composition except for a different fat body enriched with egg lipids and evening primrose oil (Pre-Aptamil with Milupan; n = 12) with 0.2% (of fatty acids) γ-linolenic acid (GLA), 0.4% AA, and 0.3% DHA. Physical examinations, anthropometric measurements, and collections of venous blood samples were performed at the ages of 5 d and 1, 2, 3, and 4 mon (24).

In a third trial, we studied four full-term infants with newly diagnosed phenylketonuria detected by newborn screening, who thrived well and had no other apparent disturbance of health. The infants were studied at a postnatal age of 18 ± 3 days (M ± SD) with a normal body weight for age (3.3 ± 0.6 kg). Three infants had been fully breast-fed and one had been formula-fed. Blood samples were obtained before and at defined intervals over four days after the time of introduction of a therapeutic phenylalanine-free diet, in which all fat was contributed by corn oil. This diet provided an LA intake of 12–16 g per day, but no other n-6 PUFA. Corn oil has a slightly higher natural enrichment of ^{13}C than other dietary fats commonly used in Europe (25), including European HM or formula.

Analytical methods. For the analyses of plasma phospholipid fatty acids, lipids were extracted from plasma into chloroform/methanol, lipid classes were separated by thin-layer chromatography and fatty acids transesterified with methanol under acid catalysis as previously described (26). Fatty acid methyl esters were determined by high-resolution capillary gas–liquid chromatography with confirmation of peak identification by mass spectrometry, as reported in detail elsewhere (27). A gas chromatograph (HP 5890 A; Hewlett Packard, Palo Alto, CA) coupled *via* a combustion interface to an isotope ratio mass spectrometer (Delta S; Finnigan MAT, Bremen, Germany) was used for isotope ratio analyses of fatty acid methyl esters (28). ^{13}C-contents are expressed as $\delta^{13}C$-values relative to PeeDeeBelemnite carbonate as defined by Craig (29). The mean standard deviation for determination of $\delta^{13}C$-values of physiologically important fatty acid methyl esters from plasma samples was 0.5 per thousand (‰) (28). Minitab™ (Minitab Inc., State College, PA) was used for statistical analysis with unpaired and paired *t*-tests.

RESULTS

Plasma phospholipid ω6 PUFA in full-term infants fed human milk or LC-PUFA–free formula. There was no significant change of LA contributions to plasma phospholipids in breast-fed infants between the ages of two and eight weeks (Table 1). In contrast, LA values increased significantly in infants fed formula between the ages of two weeks and one month, and were significantly higher than in breast-fed infants both at the ages of one and two months. Values of GLA did not differ between the two groups throughout the study. After two weeks of age, dihomo-GLA (DGLA) values increased significantly in both groups; and breast-fed infants showed significantly higher DGLA values than infants fed formula at the age of two months (Table 1). AA values were significantly higher in infants fed human milk than in those receiving formula throughout the study. Breast-fed infants maintained relatively stable plasma phospholipid AA contents during the first two months of life, whereas percentage contributions of AA decreased significantly in infants fed formula between the ages of two weeks and two months. There was no difference between the groups in gains of weight and head and chest circumference, but longitudinal growth was significantly greater in formula-fed than in breast-fed infants (1.01 ± 0.22 vs. 0.74 ± 0.24 cm/wk, $P < 0.05$).

TABLE 1
ω6 Polyunsaturated Fatty Acids in Plasma Phospholipids of Full-Term Infants Fed Human Milk or Formula[a]

Age	Two weeks	One month	Two Months
Linoleic acid (C18:2n-6)			
Human milk	19.3 ± 1.66	16.52 ± 1.72[c]	17.25 ± 1.79[b]
Formula	20.76 ± 09.70[d]	22.91 ± 0.52[c,d]	23.45 ± 1.01[b]
γ-Linolenic acid (C18:3n-6)			
Human milk	0.08 ± 0.02	0.10 ± 0.02	0.15 ± 0.02
Formula	0.13 ± 0.03	0.11 ± 0.02	0.09 ± 0.01
Dihomo-γ-linolenic acid (C20:3n-6)			
Human milk	2.36 ± 0.15[d]	2.82 ± 0.17	3.40 ± 0.19[b,d]
Formula	2.66 ± 0.19[d]	3.13 ± 0.21[d]	2.77 ± 0.21[b]
Arachidonic acid (C20:4n-6)			
Human milk	9.39 ± 1.00[b]	9.06 ± 1.04[b]	8.41 ± 1.19[b]
Formula	6.91 ± 0.38[b,d]	6.16 ± 0.35[b]	5.74 ± 0.37[b,d]

[a]% w/w, mean ± SEM. Identical superscripts indicate significant differences: [b]= $P < 0.05$, [c]= $P < 0.01$ between groups (unpaired *t*-test); [d]= $P < 0.05$ between different time points within one group (paired *t*-test).

Plasma phospholipid ω6 PUFA in full-term infants fed formula without and with preformed dietary LC-PUFA. In both feeding groups, values of LA and GLA increased between the ages of five days and one month, whereas percentage contributions of AA decreased. At the ages of 1, 2, and 3 mon, percentage contributions of LA were significantly higher in infants fed LC-PUFA–free formula than in those receiving formula with preformed LC-PUFA (Table 2). Neither GLA nor DGLA values differed between the two groups throughout the study. From the age of one month onward, percentage contributions of AA were significantly higher in infants receiving formula enriched with LC-PUFA than in those fed LC-PUFA–free formula. Both formulas were well tolerated, and there was no difference between groups in gains of weight, length, and head circumference. Throughout the study, neither retinol nor α-tocopherol plasma concentrations differed between feeding groups (24).

TABLE 2
ω6 Polyunsaturated Fatty Acids in Plasma Phospholipids of Full-Term Infants Fed Formula without (F, n = 10) and with Arachidonic and Docosahexaenoic Acids (LCP-F, n = 12)[a]

Age	Five days	One month	Two months	Three months	Four months
Linoleic acid (C18:2n-6)					
Formula	15.17 ± 1.05[d]	25.63 ± 0.82[b,d]	25.99 ± 0.70[b,c]	27.26 ± 0.83[b,c]	24.82 ± 1.53
LCP-F	14.74 ± 1.11[d]	22.09 ± 0.39[b–d]	22.78 ± 0.54[b]	22.82 ± 0.88[b]	24.48 ± 0.79[c]
γ-Linolenic acid (C18:3n-6)					
Formula	0.01 ± 0.01[d]	0.12 ± 0.02[d]	0.10 ± 0.02	0.18 ± 0.09	0.10 ± 0.03
LCP-F	0.09 ± 0.02[d]	0.13 ± 0.01[d]	0.10 ± 0.02	0.11 ± 0.02	0.12 ± 0.01
Dihomo-γ-Linolenic acid (C20:3n-6)					
Formula	2.86 ± 0.23	3.09 ± 0.23	3.29 ± 0.32	3.02 ± 0.21	3.18 ± 0.39
LCP-F	2.88 ± 0.18	2.97 ± 0.11[c]	2.74 ± 0.10[c]	2.80 ± 0.13	2.76 ± 0.07
Arachidonic acid (C20:4n-6)					
Formula	14.15 ± 0.82[d]	7.60 ± 0.52[b,d]	6.95 ± 0.42[b]	7.22 ± 0.45[b]	6.68 ± 0.43[b]
LCP-F	14.86 ± 1.03[d]	9.74 ± 0.32[b–d]	9.57 ± 0.31[b]	9.24 ± 0.21[b,c]	8.61 ± 0.30[b]

[a]% w/w, mean ± SEM; $P < 0.01$. Identical superscripts indicate significant differences: [b]= $P < 0.05$ between groups; [c]=$P < 0.05$, [d]= $P < 0.0001$ between ages; LCP-F, formula with LC-PUFA.

Estimation of AA synthesis in full-term infants by using isotopic analyses. Analysis of HM samples from two of the three breastfeeding mothers revealed LA contents of 10.7 and 11.7% with δ^{13}C-values of −32.6 and −30.7 per thousand (‰), respectively, and the formula used contained 12.8% LA with a δ^{13}C-value of −29.8‰. In contrast, the corn oil used in the therapeutic diet of all infants contained 58.1% LA with a δ^{13}C of −16.4‰. Following the dietary change with introduction of corn oil, ^{13}C-enrichment of plasma LA increased rapidly in all infants (Fig. 1). The steepest increase was observed within the first 24 h after diet change, with a smaller rise of δ^{13}C over baseline thereafter. Although corn oil does not contain any appreciable DGLA and AA, there were also clear increases of ^{13}C-enrichment of plasma DGLA on days 1 and 4 and of plasma AA on day 4 (Fig. 1), but the change of δ^{13}C over baseline was clearly smaller in AA than in DGLA. These results indicate active endogenous synthesis of DGLA and AA in full-term infants in the first month of life. We used a simplified isotope balance equation described in detail elsewhere (28) to estimate the contribution of dietary LA to total serum LA and the contribution of AA synthesized from plasma LA to total plasma AA. The calculated proportions of serum LA contributed by dietary LA were 60.8 ± 7.4% (M ± SEM) on day 1 and 83.8 ± 4.0% on day 4. Proportions of serum AA contributed by endogenous LA conversion were 6.0 ± 6.0% on day 1 and 23.3 ± 4.3% on day 4.

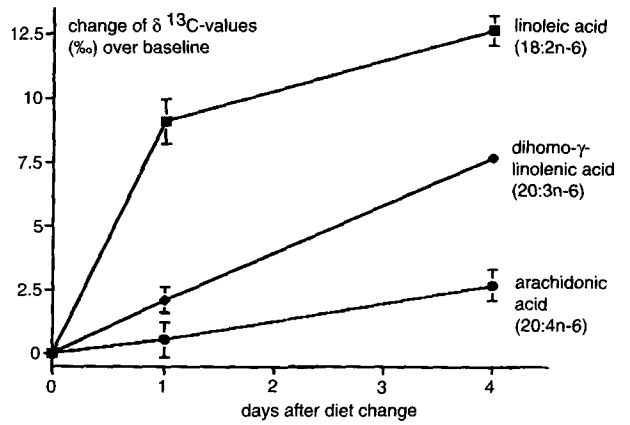


FIG. 1. Change of ^{13}C-enrichment [δ^{13}C over baseline, per thousand (‰), mean ± SEM] of plasma ω6 fatty acids in four newborn infants one and four days after dietary change from human milk or formula to a therapeutic formula with all fat contributed by corn oil, which has a higher natural ^{13}C-enrichment than most dietary fats in Europe. Values for dihomo-γ-linolenic acid represent means and SEM of only two infants. The increase of ^{13}C-enrichment of dihomo-γ-linolenic acid and arachidonic acid proves active endogenous synthesis *in vivo*.

DISCUSSION

The quality and composition of dietary fat supplied to infants has important structure and functional effects on their rapidly growing tissues (30). Among the many different aspects regarding the optimal lipid supply with HM and infant formulas, the dietary intake and metabolism of LC-PUFA receive increased attention. The results presented here indicate that full-term infants fed formula without AA and DHA are unable to match the AA status of infants fed HM for at least two months after birth, even though they receive a generous supply of the precursor fatty acids in amounts similar to HM. These data do not give any indication for a marked maturation of LC-PUFA synthesis activity in the first two months after birth. Compared to the breast-fed group, the formula-fed infants show a relatively less pronounced depletion of AA (at two months 68% of the mean of breast-fed infants) than of DHA (55%) (23), which may reflect the more complex synthesis of DHA and/or a difference in turnover rate.

It is conceivable that introduction of preformed LC-PUFA into infant formula similar to the biological model of HM

might be advantageous for the recipient infant (31). We have shown here that enrichment of an infant formula with preformed AA and DHA in amounts typical for mature HM improves AA status of full-term infants, and phospholipid AA values matching those of breast-fed infants are achieved up to the age of four months. Thus, LC-PUFA in the form used in this formula are effectively absorbed and metabolized. Although the enriched formula did contain 0.2% GLA, we did not find increased values of GLA and its elongation product, DGLA, in plasma phospholipids of the recipient infants. It appears that the contribution of relatively small amounts of dietary GLA to infantile phospholipid PUFA is minor. In these studies with a limited number of infants, there was no relation of AA supply and plasma lipid contents to clinical outcome parameters, such as growth. Hence, there are no data to address the question of a possible biological advantage of adapting dietary AA intake to that of breast-fed infants. No adverse side effects were observed, and there was no reduction of plasma levels of lipid-soluble antioxidant vitamins (24), which were in the reference range reported before for young infants (32).

In our stable isotope studies utilizing small natural variations of ^{13}C-enrichment in dietary lipids, gas chromatography combustion isotope ratio mass spectrometry proved to be a highly sensitive and precise tool for studying PUFA metabolism in infants. Our results demonstrate active AA synthesis in full-term neonates *in vivo*. These findings are in accordance with a previous report on demonstrable enzyme activities of Δ6 and Δ5-desaturases in liver microsomes of deceased neonates (33). However, the activity of synthesis is rather low, and apparently only about 6% of total plasma AA is renewed by this pathway per day. Whether this activity is sufficient to provide adequate amounts of AA to support rapid tissue growth with physiological composition remains highly questionable. Rather, full-term infants fed formula may require a dietary supply of some preformed AA if the biochemical status of breast-fed infants is to be achieved. Further studies are needed to explore the effects of variations in dietary composition on endogenous LC-PUFA synthesis, as well as possible clinical effects of variations in AA status. Such studies may contribute to establishing the n-6 and n-3 PUFA requirements of formula-fed, full-term infants.

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A Randomized Trial of Visual Attention of Preterm Infants Fed Docosahexaenoic Acid Until Two Months[1]

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ABSTRACT: This was a randomized, double-blind trial to determine if a nutrient-enriched (preterm) formula supplemented with 0.2% docosahexaenoic acid (DHA, 22:6n-3) from a low eicosapentaenoic acid (0.06%) source of marine oil would enhance visual novelty preference and attention of preterm infants. Both the standard and experimental formulas contained 3% of total fatty acids as linolenic acid (18:3n-3) and were fed from approximately three days of age to two months past term. After two months, both diet groups were fed a commercially-available term formula with linolenic acid as the only source of n-3 fatty acid. At 12 mo visual recognition memory (novelty preference) and visual attention (number and duration of discrete looks) were determined with the Fagan Test of Infant Intelligence. The DHA-supplemented group compared with the control group had more and shorter duration looks in comparisons of familiar and novel stimuli, confirming earlier evidence that DHA can increase information processing speed of preterm infants who otherwise are receiving good intakes of linolenic acid. Because supplementation was stopped at two months and the effects seen at 12 mon, this study demonstrates for the first time that a relatively short period of DHA supplementation can produce significant effects on later visual attention.
Lipids 31, 85–90 (1996).

Earlier we tested the hypothesis that preterm infants fed an infant formula supplemented with a marine oil source of docosahexaenoic acid (DHA) would have enhanced visual and neural development compared with infants fed linolenic acid as their sole source of n-3 fatty acids. The DHA-supplemented group had higher preferential looking grating acuity at two and four months (1,2) and more (3,4) and shorter duration looks (4) during paired comparisons of novel and familiar stimuli at 6.5, 9, and 12 mon. Both higher visual acuity (5) and shorter look duration during paired comparisons of familiar and novel stimuli (6) are analogous to observations made in monkey infants with better DHA status, i.e., those fed linolenic acid-replete compared with linolenic acid-deficient diets. A major difference between the monkey infant studies and our randomized trial in preterm infants, however, was that the group of infants with poorer DHA status were fed good intakes of linolenic acid, i.e., 1.2 and 2.4% of energy intake before and after two months of age, respectively.

Although these data suggested that DHA was conditionally essential for normal visual and neural outcomes of preterm infants, growth, another planned outcome of the trial, was adversely affected by long-term DHA supplementation (7). DHA supplementation decreased plasma and red blood cell (RBC) phospholipid arachidonic acid (AA) (20:4n-6) (8), and plasma phosphatidylcholine AA, a marker of AA status, was highly correlated with first-year growth (9). The current trial provided the DHA-supplemented group with a lower eicosapentaenoic acid (EPA, 20:5n-3) source of marine oil DHA for a shorter period of time than our first trial (4), with the intent of minimizing the effect of n-3 long-chain polyunsaturated fatty acids on AA status and growth (10). As a result, the experimental group in this study received formula with the same amount of DHA (0.2%) but with only half as much long-chain n-3 fatty acid (0.26% instead of 0.5%). Infants were fed their assigned formulas until two months past term, and the effects of DHA supplementation on visual attention later in infancy are reported here.

EXPERIMENTAL PROCEDURES

Subject selection. Fifty-nine infants randomly assigned to treatment constituted the final study population in this, our second, double-blind study of the effects of DHA supplementation on preterm infants. The selection criteria for infants in the total sample have been reported in detail (10). Briefly, this was a group of infants weighing between 747 and 1275 g at birth who met the strict criteria for eligibility used in our earlier trial (4), except for having an incidence of bronchopulmonary dysplasia (BPD) or chronic lung disease of ~40%, typical of infants in this birth weight range. The diagnosis of BPD was based on a continuous requirement for supplemental oxygen at 28 d of age and the presence of characteristic radiologic signs of BPD. At 12 mon, a subset of 27 of these infants (12 control, 15 DHA-supplemented) received the same version of the Fagan Test of Infant Intelligence administered in our earlier trial. Their results are included in this report, and their neonatal/perinatal characteristics are shown in Table 1.

[1]Based on a presentation at the AOCS Annual Meeting & Expo in San Antonio, Texas, May 7–11, 1995.

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Abbreviations: AA, arachidonic acid; BPD- bronchopulmonary dysplasia; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PE, phosphatidylethanolamine; PMA, postmenstrual age; RBC, red blood cell.

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TABLE 1
Neonatal and Perinatal Characteristics of Infants Randomly Assigned to Receive Commercially Available Formulas (control) or These Formulas with Added Marine Oil–Docosahexaenoic Acid (DHA-supplemented)

Variable	Control (n = 12)	DHA-supplemented (n = 15)
Birth weight (g)	1050 ± 149[a]	1027 ± 153
Gestational age (wk)	28.2 ± 1.5	27.9 ± 1.5
Black/white	10/2	15/0
Male/female	7/5	8/7
Apgar at 5 min	7.0 ± 1.6	7.1 ± 1.4
Gravida (number)[a]	3.1 ± 1.2	2.5 ± 1.2
Mother's education (yr)	11.9 ± 1.4	12.0 ± 1.0
Father's education (yr)	12.0 ± 1.4	12.2 ± 1.3
Ventilator (h)[b]	72 ± 5	65 ± 10
Total oxygen (h)[b]	261 ± 6	154 ± 9
First formula (h)	86 ± 72	92 ± 33
Age at enrollment (d)	3 ± 1	3 ± 1
Weight at enrollment (d)	1001 ± 189	965 ± 160
Two-month RBC PE DHA[c,d]	3.9 ± 0.7	7.6 ± 0.9
Weight at two months (kg)	4.93 ± 0.8	4.56 ± 0.5

[a]Mean ± SD.
[b]Because ventilator and total oxygen were not normally distributed, the individual values were transformed to $\log_{10}$ to approximate a normal distribution. The log hours ($X \pm$ SD) were calculated for each dietary group and the antilog taken to reach $X \pm$ SD (h).
[c]Red blood cell phosphatidylethanolamine (RBC PE) DHA at two months as a percent of total fatty acids (g/100 g).
[d]$P < 0.05$ for a difference between diet groups.

TABLE 2
Formula Fatty Acid Composition (g/100 g total fatty acids)[a]

	Preterm formula		Term formula
	Control	DHA-supplemented	
Fatty acid	3 d to 2 mon	3 d to 2 mon	2–12 mon
8:0	28.1	28.0	2.6
10:0	15.9	15.7	2.1
12:0	7.8	7.8	17.5
14:0	3.3	3.3	7.2
16:0	6.5	6.5	10.2
18:0	3.2	3.2	4.2
18:1	11.0	11.0	16.1
18:2n-6	21.2	21.2	34.3
18:3n-3	2.4	2.4	4.8
20:5n-3	—	0.06	—
22:6n-3	—	0.2	—

[a]The preterm formulas contained 812 kcal/L and were fed until two months past expected term when infants weighed ~4.8 kg. All infants received an unsupplemented term formula with 676 kcal/L from 2 until 12 mon past expected term. Abbreviation as in Table 1.

Experimental design. Consent for the study was obtained according to a protocol approved by the Institutional Review Board of The University of Tennessee, Memphis. Only parents who stated that they planned to feed infant formula were approached for permission to enroll their infant in this study. Infants were randomly assigned to a diet group by opening the next available envelope assigned to their gender. Each envelope contained a number identifying the formula, and the randomization was done in sequential series of eight. Each formula-gender appeared twice in each set of eight envelopes. The infants were enrolled when they were ready to start enteral feeding between two and five days of age. A preterm formula (Similac Special Care; Ross Products Division, Abbott Laboratories, Columbus, OH) was fed to the control group. The experimental formula was prepared by substituting a small amount of marine oil for an equivalent amount of the fat blend in the preterm formula. Otherwise, the experimental formula was identical to the commercially available preterm formula. Compared with most sources of marine oil, the source of marine oil added to the experimental formula had a relatively high ratio of DHA to EPA. The fatty acid composition of the two preterm formulas is shown in Table 2.

The preterm formula was designed especially for the higher nutrient needs of preterm as compared with term infants. Both diet groups received a low-iron version of the formula containing 24 kcal/oz (11) with or without a low EPA source of marine oil from enrollment until 2 mon past term. Infants received a daily supplement of 5 mg of iron from ferrous sulfate, beginning when they weighed 1.8 kg body weight and ending when they were two months past term. From 2 to 12 mon, infants from both groups were provided with a commercially-available formula (Similac with Iron) (11) designed for the nutrient requirements of infants born at term. The formulas were provided free to parents and without restriction. Compared with a group of ~60 term infants in the same hospital, the preterm infants in this study at 12 mon had slightly lower normalized weights but identical normalized lengths (12), evidence that they were well-nourished.

The gestational age of each infant was determined at birth by a combination of obstetric and pediatric assessments. The 52-wk (12 mon) Fagan Test was administered at 92 wk postmenstrual age (PMA) (12 mon) to all who returned for this visit.

Visual behavior testing. The method used to measure visual recognition memory and attention at 12 mon was the same as has been described (4). All tests were done by the same investigator, who was unaware of the infants' diets at the time of testing.

Statistical methods. This study was designed to test several hypotheses, including the hypothesis that visual novelty preference and attention of preterm infants during paired comparisons of novel and familiar stimuli would be enhanced by DHA supplementation. The power analysis for the trial was based on the number of infants needed to reject the null hypothesis for an effect of DHA supplementation on growth. Based on this analysis, 30 infants per group were planned.

At 12 mon, approximately half of the infants in each diet group were tested with a newer version of the Fagan Test that yielded data for look duration and number significantly different from the older version of the Fagan Test (Carlson, S.E., unpublished data). Because only the results obtained with the older version of the Fagan Test could be compared directly with those from our earlier study, only the scores of infants tested with that version are reported in Tables 3 and 4, where they are contrasted with results from that study. Many comparisons between diets (Table 4) suggest the null hypothesis

TABLE 3
Total Number of Discrete Looks During Familiarizations by Infants Fed a Commercial Formula (control, n = 12) or the Same Formula with a Marine Oil Source of DHA[a]

	Diet	12 mon	12 mon[b]
Total looks (#)	Control	37.1 ± 2.1	38.4 ± 1.6
	DHA-supp.	38.9 ± 1.5	38.9 ± 1.7
Looks left (#)	Control	19.3 ± 1.2	20.2 ± 1.1
	DHA-supp.	21.1 ± 1.1	19.4 ± 1.1
Looks right (#)	Control	17.8 ± 1.1	18.2 ± 0.9
	DHA-supp.	17.8 ± 0.7	19.5 ± 1.0
Time/total look (s)	Control	1.41 ± 0.08	1.39 ± 0.06
	DHA-supp.	1.33 ± 0.06	1.34 ± 0.06
Time/look left (s)	Control	1.37 ± 0.10	1.36 ± 0.08
	DHA-supp.	1.25 ± 0.08	1.37 ± 0.08
Time/look right (s)	Control	1.48 ± 0.09	1.54 ± 0.07
	DHA-supp.	1.45 ± 0.06	1.37 ± 0.08

[a]DHA-supplemented, n = 15. Mean ± SEM; supp., supplemented; (#), number; (s), second.
[b]Twelve-month looks and look duration in infants fed control and DHA-supplemented formula in our earlier randomized trial (Ref. 4).

cannot be accepted because of Type II error that resulted from the unplanned loss of power.

The effects of diet on 12-mon visual attention were determined by Student's *t*-test. A *post hoc* two-way analysis of variance for diet and BPD was used to look for an effect of BPD, as well as diet. These analyses were done on a MacIntosh IIcx computer with StatView software (Abacus Concepts, Inc., Berkeley, CA). A P value of <0.05 was considered statistically different.

TABLE 4
Visual Attention During Paired Comparisons by Infants Fed a Commercially Available Formula (control, n = 12) or the Same Formula with a Marine Oil Source of Docosahexaenoic Acid[a]

		12 mon	12 mon[b]
Novel time (% total)	Control	64.0 ± 1.9	64.6 ± 1.2
	DHA-supp.	59.7 ± 1.7	60.5 ± 1.3[c]
Total time (s)	Control	50.0 ± 1.6	48.9 ± 1.4
	DHA-supp.	50.8 ± 1.7	51.2 ± 1.4
Time to novel (s)	Control	33.1 ± 1.4	32.6 ± 1.2
	DHA-supp.	31.5 ± 1.5	31.9 ± 1.2
Time to familiar (s)	Control	16.9 ± 1.0	16.3 ± 0.8
	DHA-supp.	19.3 ± 0.9	19.3 ± 0.9[c]
Total looks (#)	Control	40.4 ± 2.7	42.4 ± 1.3
	DHA-supp.	46.8 ± 2.7	47.7 ± 1.4[d,e]
Looks to novel (#)	Control	22.9 ± 1.5	23.6 ± 0.8
	DHA-supp.	25.3 ± 1.6	26.0 ± 0.8[c,e]
Looks to familiar (#)	Control	17.5 ± 1.4	18.8 ± 0.8
	DHA-supp.	21.5 ± 1.3[c]	21.7 ± 0.8[c,e]
Time/look (s)	Control	1.30 ± 0.10	1.18 ± 0.05
	DHA-supp.	1.13 ± 0.07	1.11 ± 0.05[e]
Time/novel look (s)	Control	1.49 ± 0.09	1.43 ± 0.07
	DHA-supp.	1.28 ± 0.06[c]	1.27 ± 0.07[e]
Time/familiar look (s)	Control	1.04 ± 0.11	0.85 ± 0.05
	DHA-supp.	0.95 ± 0.08	0.91 ± 0.05

[a]DHA-supplemented, n = 15. Mean ± SEM. Abbreviations as in Table 3.
[b]Visual attention at 12 mon in infants fed control and DHA-supplemented formula in an earlier trial (Ref. 4).
[c]Different from control infants of the same study age, $P < 0.05$ by Student's *t*-test.
[d]Different from control infants of the same study age, $P < 0.01$.
[e]Diet effect ($P < 0.05$) with repeated measures analysis of variance including tests at 6.5, 9, and 12 mon.

RESULTS

Age at test. The actual test ages in weeks from term (mean ± SD) and the numbers of infants tested with the original 52-wk version of the Fagan Test in each diet group were, respectively, 52.6 ± 1.2 (n = 12) for the control group and 52.4 ± 1.0 (n = 15) for the DHA-supplemented group. This age is described throughout the paper as 12 mon. The mean test age of the diet groups did not differ.

Neonatal and perinatal characteristics of the infants. The infants in each diet group are described in Table 1. The groups were similar in all respects except that the group fed DHA-supplemented formula had higher RBC phosphatidylethanolamine (PE) DHA at two months compared with the control group as a result of receiving a DHA-supplemented diet from birth until that age. After the DHA supplement was discontinued at two months, RBC PE DHA (g/100 g total fatty acid) declined progressively over the next few months. At 12 mon, the groups could not be distinguished from each other on the basis of their RBC PE DHA (10).

Effect of diet on visual attention during familiarization. During familiarization, infants in the two diet groups had similar numbers of looks at each study age, and the total number of looks was divided nearly equally between the infants' left and right (Table 3). Both the number of looks and look duration at 12 mon were similar to the results of our earlier randomized trial (Table 3).

Infants with BPD differed from those without BPD in that they had significantly fewer total looks during familiarization with the Fagan Test (Table 5, $P < 0.02$). Because all infants were required to look for the same amount of time during familiarization, a lower number of looks was directly related to a significantly longer look duration in infants with BPD compared with infants without BPD ($P < 0.01$).

Effect of diet on visual attention during paired comparisons. Both diet groups had a significant preference for the novel stimuli during paired comparison tests (Table 4), but DHA-supplemented compared with control infants had significantly more discrete looks to the familiar stimuli during paired comparison testing ($P < 0.05$). A comparison of the 12-month data from this trial with that from our earlier trial (shown in the last column of Table 4) indicates similar increases in the numbers of total looks, looks to novel, and looks to familiar with DHA supplementation, and remarkably similar looking times and novelty preferences. At 12 mon the average time/novel look (s) was lower in DHA-supplemented infants ($P < 0.05$), and the average time/look (s) tended in the same direction. Again, the average times/novel look for the control and DHA-supplemented infants in this study were practically the same as for their respective groups in our earlier study (Table 4).

At 12 mon, infants who developed BPD compared with those without BPD had a longer average time/look ($P < 0.03$),

average time/novel look ($P < 0.02$), and average time/familiar look ($P < 0.06$) (Table 5). Although the numbers in each diet-BPD group were small, longer look duration in infants with BPD appeared to be due to higher look duration in the BPD infants fed control formula because the means for the DHA-supplemented infants with and without BPD were similar. Novelty preference determined during paired comparison tests was not affected by BPD (Table 5).

DISCUSSION

While subjects of this study compared with our earlier study (4) had similar birth weights and gestational ages and were fed experimental and control formulas with the same amounts of DHA and linolenic acid, respectively, the studies also differed in several ways: (i) The source of marine oil was lower in EPA in the second compared with the first study (0.06 vs. 0.3%). (ii) The experimental formula with DHA was fed until two months instead of nine months past term. (iii) Infants in the second randomized trial were fed a nutrient-enriched, energy-concentrated (24 kcal/oz) preterm formula instead of a 20 kcal/oz term formula from 36 to 48 wk PMA. (iv) The infants in the second trial had a 40% incidence of BPD, usual for this weight range, whereas practically no infants with BPD were included in our first study (3 of 67) (7).

This study confirmed that preterm infants fed DHA have shorter look durations to novel stimuli on paired comparison tests compared with infants fed linolenic acid as their sole source of n-3 fatty acid. In addition, the outcomes for visual recognition memory and attention (number and duration of looks) of control and DHA-supplemented infants were numerically similar to the responses observed earlier despite the smaller than intended sample size and several differences in design that could have decreased the response of the experimental group (Table 4).

For example, the shorter period of DHA supplementation might have been expected to decrease or to eliminate the effect of DHA supplementation on number and duration of discrete looks during the test. This did not appear to happen.

Because the effects of DHA supplementation on visual attention were found ten months after DHA was removed from the diet, the data suggested that the early diet had a long-term effect on the neural pathways responsible for differences in look duration. The idea is plausible, given that human milk feeding, which provides DHA, has been shown to increase DHA in the central nervous system compared with DHA-free infant formulas (13,14).

What cannot be concluded from this study is whether or not this short interval of DHA supplementation interacted with the prolonged feeding of nutrient-enriched formula to produce the effects of short-term DHA feeding on 12-month look duration shown here. A comparison of the outcomes at 12 mon for control infants without BPD in this study (Table 5) with controls in our earlier study suggests, however, that prolonged use of a nutrient-supplemented formula in this trial neither enhanced novelty preference nor shortened look duration.

Among preterm infants, it is well known that those with BPD are at higher risk for poorer neurodevelopmental outcomes than those without BPD. The reasons for this are not entirely clear. Because novelty preference has predictive validity for performance on standardized intelligence tests in childhood (15,16), we might have anticipated that infants with BPD would have had a lower novelty preference than those without BPD. Instead, the infants with BPD in this study had the same visual recognition memory (novelty preference) as infants without BPD (Table 5).

Compared with infants without BPD, however, infants with BPD had longer looks during the paired comparison (novelty) tests. When the look durations to the novel stimuli and familiar stimuli were considered independently, BPD infants also had significantly longer duration looks to both novel stimuli and familiar stimuli. The data in Table 5 suggest that the higher look duration seen in the analysis of variance was due to a higher look duration among infants with BPD fed the control diet. Infants with BPD who received the DHA-supplemented diet appeared to have the same look du-

TABLE 5
Effect of DHA Supplementation on 12-Month Novelty Preference and Visual Attention in Preterm Infants without or with Bronchopulmonary Dysplasia (BPD)

	Control		DHA-supplemented		Diet	BPD	Diet × BPD
Infant Looking at objects during test	No BPD (n = 7)	BPD (n = 5)	No BPD (n = 9)	BPD (n = 6)			
Familiarization							
Total Looks	40.6 ± 1.9[a]	32.2 ± 3.2	40.3 ± 1.8	36.7 ± 2.6	N.S.[b]	$P < 0.02$	N.S.
Time/look (s)	1.26 ± 0.06	1.62 ± 0.14	1.27 ± 0.06	1.41 ± 0.11	N.S.	$P < 0.01$	N.S.
Paired comparisons							
Novelty time (%)	64.9 ± 2.2	62.6 ± 3.5	59.2 ± 2.3	60.4 ± 2.5	N.S.	N.S.	N.S.
Total looks (#)	42.8 ± 3.0	37.0 ± 4.9	46.2 ± 2.6	47.7 ± 6.1	$P < .10$	N.S.	N.S.
Looks to novel (#)	24.6 ± 2.0	20.6 ± 2.2	24.9 ± 1.6	25.8 ± 3.4	N.S.	N.S.	N.S.
Looks to familiar (#)	18.3 ± 1.4	16.4 ± 2.8	21.3 ± 1.1	21.8 ± 2.9	$P < 0.04$	N.S.	N.S.
Time/look (s)	1.12 ± 0.05	1.55 ± 0.19	1.11 ± 0.07	1.16 ± 0.13	$P < 0.07$	$P < 0.03$	N.S.
Time/novel look (s)	1.32 ± 0.05	1.73 ± 0.17	1.25 ± 0.07	1.32 ± 0.11	$P < 0.02$	$P < 0.02$	N.S.
Time/familiar look (s)	0.85 ± 0.05	1.31 ± 0.22	0.94 ± 0.09	0.98 ± 0.16	N.S.	$P < 0.06$	N.S.

[a]Mean ± SEM. Abbreviations as in Table 3.
[b]N.S., not significant.

ration as DHA-supplemented infants without a history of BPD. Larger numbers of infants would need to be studied to determine if this is the case.

The familiarization interval for the Fagan Test is long and appears to be insensitive to known differences between groups of infants, e.g., the longer period of familiarization needed by preterm compared with term infants to demonstrate a novelty preference (17). Just as we found earlier (4), dietary DHA increased look number and decreased look duration during paired comparisons but not during familiarizations. Infants with BPD compared with those without BPD, however, had significantly fewer looks (and the reciprocal, longer look duration) during familiarizations, regardless of diet, suggesting poorer ability to shift attention.

In summary, the data from this randomized trial confirmed an earlier report (4) that preterm infants fed DHA have shorter look duration during paired comparison tests than infants fed linolenic acid as their sole dietary source of n-3 fatty acids. These data are analogous to those from monkey infants fed diets with, compared to without, adequate linolenic acid in that the group with better DHA status had a shorter look duration (6). Like novelty preference, shorter visual fixation in infancy is related to superior performance in infancy and childhood (18–20). Longer look duration has been suggested to be evidence of slower visual information processing (21,22), slower ability to disengage attention (23), or increased reactivity. If this is so, preterm infants fed DHA for only a short interval after birth may have more rapid visual information processing or more mature attention months after DHA is removed from their diet.

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A Randomized Trial of Visual Attention of Preterm Infants Fed Docosahexaenoic Acid Until Nine Months[1]

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ABSTRACT: This randomized, double-blind trial tested the hypothesis that the addition of 0.2% docosahexaenoic acid (DHA, 22:6n-3) from marine oil to commercially-available preterm and term formulas with ≥3% linolenic acid (18:3n-3) would enhance novelty preference and visual attention of preterm infants. Among preterm infants cared for in our center, study infants were a select group considered to be at lower risk for developmental delay. Study infants received their assigned diet (control, DHA-supplemented) from a mean postnatal age of 25 d until 9 mon past term. At 6.5, 9, and 12 mon past term, they were tested for visual recognition memory (novelty preference) and attention with the Fagan Test of Infant Intelligence. The effects of DHA supplementation were analyzed by repeated measures analysis of variance. In paired comparisons of novel and familiar stimuli, DHA-supplemented and control infants had the same novelty preference, but supplemented infants had more discrete looks to both novel ($P < 0.03$) and familiar ($P < 0.02$) stimuli and a shorter overall look duration ($P < 0.03$). These data are analogous to those from n-3–deficient and n-3–fed monkeys in that the group with better DHA status had shorter overall look duration. Because shorter look duration has been associated with more rapid information processing, preterm infants fed formulas with only linolenic acid may have had slower information processing than those fed DHA.
Lipids 31, 91–97 (1996).

The n-3 fatty acid-deficient diets are known to decrease docosahexaenoic acid (DHA) in retinal (1,2) and neural (2–4) tissues of developing animals. These decreases are associated with impaired retinal responses to light (1,2) and slower than normal visual acuity development (4). Diets that are deficient in n-3 fatty acids also cause behavioral changes on perceptual-cognitive tasks, which have been interpreted both as evidence of poorer discrimination learning (5–8) and as changes in sensory thresholds and maturation and arousal (9). Recently, rhesus monkey infants (10) fed n-3–deficient diets were reported to have increased look duration to both faces and patterns compared with n-3–fed infants, evidence of slower information processing (11,12), slower disengagement of attention (13), or increased reactivity.

Because the effect of an n-3 fatty acid-deficient diet on neural tissue of developing animals is reflected in erythrocyte phospholipid DHA (2,14), and because DHA accumulates rapidly during the last intrauterine trimester (15), low erythrocyte phospholipid DHA in preterm infants fed formula with linolenic acid led us to consider that DHA might be conditionally essential for visual and neural development of these infants (16). An earlier report from this study documented higher visual acuity in the DHA-supplemented group compared with infants fed linolenic acid at 2 and 4 mon (17) past term.

This same randomized, double-blind study also tested the hypothesis that the addition of 0.2% DHA to formulas with ≥3% linolenic acid would enhance novelty preference and visual attention (specifically, an increased number and an average decrease in duration of discrete visual fixations) in the first year of life. In a preliminary report of these data (18), DHA-supplemented infants compared with control infants fed linolenic acid as their sole source of n-3 fatty acids were shown to have an increased number of discrete visual attentions or looks during paired-comparison (novelty) testing but no difference in total looking time. One possible interpretation of these data was that the DHA-supplemented infants had more mature visual attention (19,20). However, the data could not be interpreted in the context of any known effect of n-3 fatty acid deficiency on visual attention. This situation changed with the report of Reisbick *et al.* (10) that n-3–deficient monkey infants had increased look duration compared with n-3–fed monkey infants. Their observation suggested further analysis of the data. The novelty preference and visual attention data from this trial are presented here.

EXPERIMENTAL PROCEDURES

Subject selection. The 67 study infants and their selection have been described in detail elsewhere (17). Their description is summarized in Table 1. Sixty-four of these infants remained in the study after 4 mon and were studied with the Fagan Test. Study infants weighed between 748 and 1398 g at birth and were eligible for the study when they had tolerated enteral intakes >462 kJ (100 kcal) • kg body wt^{-1} • d^{-1} for 5–7 d. Exclusion criteria included a need for mechanical ventilation at that time, intraventricular hemorrhage > grade

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Abbreviations: ANOVA, analysis of variance; DHA, docosahexaenoic acid.

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TABLE 1
Neonatal and Perinatal Characteristics of Infants Randomly Assigned to Receive Commercially-Available Formulas (control) or These Formulas with Added Marine Oil Docosahexaenoic Acid (DHA-supplemented)[a]

Variable	Control (n = 34)	DHA-supplemented (n = 33)
Birth weight (g)	1074 ± 193	1133 ± 163
Gestational age (wk)	29 ± 2	29 ± 2
Size at birth (AGA/SGA)[b]	31/3	31/2
Black/white	32/2	26/7
Male/female	12/22	13/20
Apgar at 5 min	7.2 ± 1.7	7.0 ± 1.3
Maternal age (yr)	23 ± 6	23 ± 6
Gravida (cm)	2.5 ± 1.4	2.4 ± 1.3
Mother's formal education (yr)	11.4 ± 1.4	11.6 ± 1.6
Father's formal education (yr)	11.9 ± 1.8	11.6 ± 1.1
Ventilator (h)[c]	12 ± 7 (0–596)	6 ± 7 (0–352)
Total oxygen (h)[c]	58 ± 5 (0–830)	46 ± 5 (0–1570)
First formula (h)	78 ± 55	79 ± 54
Age at enrollment (d)	25 ± 10	24 ± 10
Weight at enrollment (g)	1304 ± 183	1330 ± 129

[a]Mean ± SD.
[b]Appropriate for gestational age (AGA)/small for gestational age (SGA). All SGA infants were between the fifth and tenth percentile of weight for age.
[c]Because ventilator and total oxygen were not normally distributed, the individual values were transformed to $\log_{10}$ to approximate a normal distribution. The log hours ($X \pm$ SD) were calculated for each dietary group and the antilog taken to reach $X \pm$ SD (h). The ranges of hours are in parentheses.

2 (21), retinopathy of prematurity > stage 2 (22), surgery for necrotizing enterocolitis, a weight less than the fifth percentile for gestational age, and a history of maternal substance abuse. These criteria were intended to eliminate potential confounding effects of uncontrolled variables such as intrauterine malnutrition, chemical insult, or chronic disease on the planned outcomes of the study.

Experimental design. The study was designed to test several hypotheses, including the hypotheses addressed in this paper, i.e., that novelty preference and visual attention would be enhanced by the addition of DHA to a formula that included the essential fatty acid, linolenic acid. The other planned outcomes of this study included biochemistry (23), visual acuity development (17), and growth (24). All growth and development measures were obtained and blood was drawn at the same follow-up visit. Blood was routinely drawn after all developmental assessments had been completed.

Consent for the study was obtained from a parent or parents according to a protocol approved by The University of Tennessee Institutional Review Board (Memphis, TN). Following this, infants were randomly assigned to a diet group by opening the next available envelope. Each envelope contained a number identifying the formula, and the randomization was done in sequential series of six. Each formula number appeared three times in each set of six envelopes. To produce the experimental formula, a small amount of marine oil was substituted for an equivalent amount of the fat blend in commercially available formulas designed for the nutrient needs of preterm infants (Similac Special Care; Ross Products Division) and term infants (Similac with Iron; Ross Products Division). The predominant fatty acids in the commercially-available formula (control) and the experimental formula (DHA-supplemented) are shown in Table 2. Study infants were fed their assigned preterm formula until discharge from the hospital at ~1.8 kg. After discharge from the hospital, they were fed their assigned term formula (control or DHA-supplemented) until 9 mon past term. Formula remained the infants' major source of nutrients and energy through at least 9 mon past expected term, but other foods were gradually added to the diet beginning at ~4 mon past term. From 9 to 12 mon most infants consumed a mixed diet that included whole cow's milk.

Visual behavior testing. The 29-wk (6.5 mon), 39-wk (9 mon), and 52-wk (12 mon) versions of the Fagan Test of Infant Intelligence (Infantest Corporation, Cleveland, OH) were administered to infants. These ages were calculated from the age the infant should have been 40 wk gestation (term). All tests were administered by the same individual (SHW) who received training by Infantest Corporation personnel in Cleveland, Ohio. She was not aware of the infants' diets at the time of testing. The version of the test used in this study was the one available from the Infantest Corporation in 1987. The test utilized a plastic viewing stage equipped with low voltage, individually housed lights with a magnifying lens attached permanently to the stage, and enclosed with a soft rubber eye cup on the side nearest the tester. The visual stimuli were black-and-white or color pictures of faces printed on washable Kimdura paper to prevent glare and to resist creases.

A Commodore 64 computer with accompanying monitor, keyboard, disk drive, and printer, and capability of accumulating real-time discrete looks of ≥0.1 s was used to record data from each discrete look during the familiarization and information processing portions of the test. The software was formatted so that time during looks to the infant's left side accumulated continuously throughout the time a "left look" key

TABLE 2
Formula Fatty Acid Composition (g/100 g total fatty acids)[a]

	Control		DHA-supplemented	
Fatty acid	Preterm formula	Term formula	Preterm formula	Term formula
8:0	28.8	2.3	28.8	2.2
10:0	14.5	2.0	14.2	2.0
12:0	10.0	17.2	9.7	16.9
14:0	4.3	7.2	4.2	7.2
16:0	6.3	9.9	6.2	10.0
18:0	3.1	4.7	3.0	4.6
18:1	9.9	17.1	9.8	17.0
18:2n-6	19.1	33.2	18.7	32.6
18:3n-3	3.0	4.8	3.1	4.9
20:5n-3	—	—	0.3	0.3
22:6n-3	—	—	0.2	0.2

[a]The preterm formulas contained 812 kcal/L and were fed until infants reached a weight of ~1.8 kg. All infants received their assigned term formula from 1.8 kg until 9 mon after term, calculated from the age they should have been 40 wk gestation. The term formula contained 676 kcal/L. See Table 1 for abbreviation.

was depressed, and time during looks to the right side accumulated continuously while a "right look" key was depressed. Each key depression/release was counted as a discrete look. A discrete look was defined as a look of ≥0.1 s duration. If an infant looked at a picture then looked away before returning his or her attention to the picture and each look was ≥0.1 s duration, two discrete looks were recorded. During the test the infant's mother was seated in a Naugahyde-covered chair with the infant on her lap. The chair was designed to comfortably hold mother and infant but narrow enough to fit between the side panels of the viewing stage. The tester was seated opposite mother and baby on a stool with a pneumatic height adjustment. The stage was placed between mother/baby and the tester and opened toward the tester so the stimuli could be placed rapidly and removed in synchrony with an audible cue from the computer.

The Fagan Test consisted of a series of familiarizations to visual stimuli (pictures of faces) followed by determinations of visual recognition memory in paired comparisons of familiar with novel stimuli. Information processing was determined from the number and duration of discrete looks during these paired comparisons. The Fagan Test has been standardized to take out age effects on novelty preference by manipulating familiarization and test times. Therefore, some age effects that depend upon familiarization criterion and available test times are meaningless or uninterpretable. As noted in the results, these include the number of looks during familiarization and the total time spent looking during paired comparisons. On the other hand, look duration automatically standardizes across various familiarization times and total looking times because it incorporates these times into the definition, so significant effects of age on look duration are meaningful.

During familiarization, infants were required to look for a fixed amount of time before proceeding to the paired-comparison (novelty) test. The time required was dependent upon the age of the infant. Younger infants (6.5 mon) were required to spend more time looking at the pictures during familiarization than older infants (9 and 12 mon). Entry of the infant's birthdate, gestational age at birth, and the date of the test automatically began the correct version of the Fagan Test. On three occasions, infants were shown two copies of the same picture simultaneously. On three other occasions, the same picture was shown on one side of the stage and then moved to the other side for accumulation of the required visual exposure. The discrete looks during these six familiarization periods were summed to equal the total number of looks during familiarization. The discrete looks to the infant's right and left sides, respectively, were also determined.

Following each familiarization, the familiar and a novel picture were placed on the stage together and the infant presented with both for a predetermined amount of time. A novelty preference score (%) was derived from the average percent of total time spent viewing the novel stimuli on ten discrete paired-comparison tests within the version of the Fagan Test administered at each age. Infants with an average score of >57% had a statistically significant novelty preference, i.e., their percent of time spent looking at the novel stimuli compared with the familiar stimuli was greater than chance. In contrast to familiarization, all infants were not required to spend the same total amount of time looking at the pictures during the paired comparisons. Instead, infants were given the opportunity to view the stimuli for a specific amount of time that was dependent on study age. Only a portion of the total time available beginning when the "left look" or "right look" key was depressed needed to be utilized for an infant to successfully complete each paired-comparison test.

Statistical methods. The power analysis for the study was based on the number of infants per group needed to reject the null hypothesis for an effect of DHA supplementation on growth, i.e., 30 infants/group. The effects of diet and study age on visual attention were determined using the General Linear Model procedure of SAS Version 6.0 (Cary, NC) (25) on the mainframe computer at The University of Tennessee, Memphis (Memphis, TN). The experimental design, involving repeated measures over time with each baby serving as its own control, may be called a split-plot or nested-factorial design (26). This analysis is particularly useful for clinical trials of the sort reported in this paper because it retains the advantages of a repeated measures analysis of variance (ANOVA), but neither estimates missing values nor excludes available data from subjects who are not tested at all ages.

Babies were nested within dietary treatments and repeatedly tested at 6.5, 9, and 12 mon. Because not all babies could be evaluated at each age, least-squares means (also called population marginal means or adjusted treatment means) were estimated for each treatment-time subclass (27). Missing values were treated as missing. Because only actual measurements were used in the analysis, the error term was probably larger than would be expected with analyses that allow estimation of missing data. At the same time, babies without assessments at every age were validly included in the analysis. The results reported here were based on 166 individual Fagan Tests: 24, 28, and 29 control infants and 27, 30, and 28 DHA-supplemented infants at 6.5, 9, and 12 mon, respectively. Of the 64 infants studied, 43 were tested at three ages, 16 at two ages, and 5 at one age. The majority of missing 6- and 9-mon values occurred in infants who reached test age before the test was available, while the majority of missing 12-mon values occurred because infants did not return for follow-up. Every available test was included in the analysis.

The specific hypotheses tested in this study were the following: (i) There is no difference in visual attention between the dietary treatments at 6.5, 9, or 12 mon. (ii) There is no change in visual attention over time for either of the two dietary treatments. To test these hypotheses, we subdivided the treatment-time sum of squares and degrees of freedom into separate contrasts and constructed F-tests. The pooled within-baby variance was used as the error term (or denominator) for F-tests of specific hypotheses at each of the time intervals and to assess changes within each dietary treatment group over time. A P value < 0.05 was considered evidence of a significant difference between compared groups.

RESULTS

Age at test. From term, the mean and SD for the actual test ages for these three versions of the Fagan Test were, respectively, 28.6 ± 2.2, 38.9 ± 1.9, and 53.0 ± 2.0 wk for the control group and 28.5 ± 2.7, 38.9 ± 2.2, and 52.7 ± 2.5 wk for the DHA-supplemented group. The mean test ages of the diet groups did not differ significantly at any study age. These study ages are described throughout this paper as 6.5, 9, and 12 mon, respectively.

Effect of diet and study age on visual attention during familiarization. During familiarization, infants in the two diet groups had similar numbers of discrete looks to pictures (Table 3). The total number of looks and the number of looks to the right and to the left were higher at 6.5 mon than at 9 and 12 mon ($P < 0.0001$), but this apparent effect of study age on numbers of looks may not be meaningful because younger infants were required to look at the pictures during familiarization for longer periods of time.

On the other hand, look duration during familiarization was significantly influenced by study age. This age effect is considered meaningful because look duration automatically standardizes across various familiarization times. Look duration decreased with age. Diet did not significantly influence look duration during familiarization, but there was a trend toward shorter look duration in DHA-supplemented infants compared with control infants (Table 3). An examination of the data shows a relatively equal distribution of total looks between the left and the right side at 6.5, 9, and 12 mon. Given the same opportunity to visually explore identical pictures on the left side and the right side of the stage, the infants as a whole did not have a preference for looking to one side or the other.

Effect of diet and study age on visual attention during paired comparisons. During novelty tests, both diet groups had a significant preference for novelty, i.e., they spent a significantly higher proportion of their total looking time viewing the novel stimuli. The ANOVA for the effects of diet and study age indicated that neither diet nor study age influenced overall novelty preference (Table 4); however, at 12 mon, DHA-supplemented infants had a lower novelty preference compared with the control group ($P < 0.05$).

On the whole, infants spent most of their available time during the tests engaged in attention to the pictures on the stage. Diet did not influence the total time spent looking at the visual stimuli during the novelty test, i.e., during the paired comparison between novel and familiar stimuli. Likewise, the actual times spent looking at novel pictures and familiar pictures were not influenced by diet (Table 4). Total looking time, the time spent looking only at novel stimuli, and the time spent looking at only familiar stimuli were higher at 6.5 mon than at 9 and 12 mon (Table 4), but this effect of study age shown by the ANOVA is meaningless given that the 6.5-mon version of the test allows more time for paired comparisons than do the 9- and 12-mon versions.

Diet influenced the number of discrete looks during the novelty test: the DHA-supplemented group had more total (novel and familiar) discrete looks compared with the control group ($P < 0.02$). This increase was also apparent when the number of looks to the novel stimuli ($P < 0.03$) and the number of looks to the familiar stimuli ($P < 0.03$) were considered independently. These increases in the number of total, novel, and familiar looks with DHA supplementation were significant at all study ages (Table 4). Because the DHA-supplemented group had more discrete looks within the same total time spent looking, they also had significantly shorter average look duration compared with the control group ($P < 0.03$). When the duration of looks to novel and familiar stimuli was considered independently, the average look duration to novel stimuli was significantly decreased by DHA supplementation ($P < 0.03$). Both diet groups had roughly similar proportions of looks to novel and familiar stimuli, so novelty

TABLE 3
Total Number of Discrete Looks and Average Look Duration During All Familiarization by Infants Fed Commercially-Available Formulas Compared with Formulas Supplemented with Marine Oil DHA[a]

					ANOVA		
	Diet	6.5 mon	9 mon	12 mon	Diet	Study age	Diet × study age
Total looks (number)	Control	52.6 ± 2.1	39.1 ± 1.8	38.4 ± 1.6			
	DHA-supp.	56.3 ± 2.0	42.0 ± 1.8	38.9 ± 1.7	N.S.	$P < 0.0001$[b]	N.S.
Looks left (number)	Control	27.4 ± 1.4	21.1 ± 1.2	20.2 ± 1.1			
	DHA-supp.	29.4 ± 1.4	22.3 ± 1.2	19.4 ± 1.1	N.S.	$P < 0.0001$[b]	N.S.
Looks right (number)	Control	25.2 ± 1.2	17.9 ± 1.0	18.2 ± 0.9			
	DHA-supp.	26.9 ± 1.1	19.6 ± 1.0	19.5 ± 1.0	N.S.	$P < 0.0001$[b]	N.S.
Time/total look (s)	Control	2.01 ± 0.08	1.49 ± 0.07	1.39 ± 0.06			
	DHA-supp.	1.84 ± 0.07	1.40 ± 0.06	1.34 ± 0.06	$P < 0.12$	$P < 0.0001$	N.S.
Time/look left (s)	Control	1.94 ± 0.10	1.39 ± 0.09	1.36 ± 0.08			
	DHA-supp.	1.80 ± 0.10	1.34 ± 0.08	1.37 ± 0.08	N.S.	$P < 0.0001$	N.S.
Time/look right (s)	Control	2.17 ± 0.10	1.68 ± 0.08	1.54 ± 0.07			
	DHA-supp.	1.96 ± 0.09	1.52 ± 0.08	1.37 ± 0.08	$P < 0.07$	$P < 0.0001$	N.S.

[a]Least squares means from repeated measures analysis of variance (ANOVA) corrected for subject error. See Table 1 for abbreviation; Supp., supplemented; N.S., not significant.
[b]The familiarization time required by the Fagan Test differed with study age. Higher numbers of looks among younger infants (6.5 mon) compared with older infants (9 and 12 mon) may not be meaningful because younger infants were required to look for longer periods of time.

TABLE 4
Visual Attention During Paired Comparisons by Infants Fed Commercially-Available Formulas Compared with Formulas Supplemented with Marine Oil DHA[a]

					ANOVA		
	Diet	6.5 mon	9 mon	12 mon	Diet	Study age	Diet × study age
Novel time (% of total)	Control	60.4 ± 1.4	62.2 ± 1.2	64.6 ± 1.2			
	DHA-supp.	59.8 ± 1.3	62.2 ± 1.2	60.5 ± 1.3[b]	N.S.	N.S.	N.S.
Total time (s)	Control	72.0 ± 1.8	51.1 ± 1.6	48.9 ± 1.4			
	DHA-supp.	72.6 ± 1.7	50.9 ± 1.5	51.2 ± 1.4	N.S.	$P < 0.0001$[d]	N.S.
Time to novel (s)	Control	45.3 ± 1.5	32.9 ± 1.3	32.6 ± 1.2			
	DHA-supp.	45.9 ± 1.5	32.6 ± 1.3	31.9 ± 1.2	N.S.	$P < 0.0001$[d]	N.S.
Time to familiar (s)	Control	26.6 ± 1.1	18.2 ± 1.0	16.3 ± 0.8			
	DHA-supp.	26.6 ± 1.1	18.3 ± 0.9	19.3 ± 0.9[b]	N.S.	$P < 0.0001$[d]	N.S.
Total looks (number)	Control	41.0 ± 1.7	40.3 ± 1.5	42.4 ± 1.3			
	DHA-supp.	48.2 ± 1.7[c]	47.0 ± 1.5[c]	47.7 ± 1.4[c]	$P < 0.02$	N.S.	N.S.
Looks to novel (number)	Control	22.2 ± 1.0	22.1 ± 0.9	23.6 ± 0.8			
	DHA-supp.	26.0 ± 1.0[c]	25.5 ± 0.8[c]	26.0 ± 0.8[b]	$P < 0.03$	N.S.	N.S.
Looks to familiar (number)	Control	18.8 ± 1.0	18.2 ± 0.9	18.8 ± 0.8			
	DHA-supp.	22.1 ± 1.0[b]	21.4 ± 0.9[b]	21.7 ± 0.8[b]	$P < 0.03$	N.S.	N.S.
Average time/look (s)	Control	1.75 ± 0.06	1.30 ± 0.06	1.18 ± 0.05			
	DHA-supp.	1.62 ± 0.06	1.13 ± 0.05[b]	1.11 ± 0.05	$P < 0.03$	$P < 0.0001$	N.S.
Time/novel look (s)	Control	2.03 ± 0.09	1.51 ± 0.08	1.43 ± 0.07			
	DHA-supp.	1.88 ± 0.08	1.33 ± 0.07	1.27 ± 0.07	$P < 0.03$	$P < 0.0001$	N.S.
Time/familiar look (s)	Control	1.42 ± 0.06	1.04 ± 0.06	0.85 ± 0.05			
	DHA-supp.	1.31 ± 0.06	0.91 ± 0.05	0.91 ± 0.05	N.S.	$P < 0.0001$	N.S.

[a]Least squares means from repeated measures ANOVA corrected for subject error. See Tables 1 and 3 for abbreviations.
[b]$P < 0.05$ compared to control infants at the same age.
[c]$P < 0.01$ compared to control at the same age.
[d]The time allowed for the novelty preference assessment differed with study age. Because younger infants (6.5 mon) were allowed more time to look than older infants (9 and 12 mon), their higher times are a meaningless age effect.

preference was related to a shorter look duration to familiar compared with novel stimuli rather than to a smaller number of looks to familiar compared with novel stimuli.

At each study age, the total number of looks and the independent looks to novel stimuli and familiar stimuli were the same despite the increased time available for viewing the pictures accorded infants who received the 6.5-mon version compared with the 9- and 12-mon versions of the test. The reason for this is apparent following an examination of the effect of study age on the look duration (total, novel, and familiar) at 6.5 mon compared with 9 and 12 mon (Table 4). In each comparison, there was a progressive decrease in look duration with increasing age. The largest decrease occurred between the ages of 6.5 and 9 mon. In every case, the look duration to familiar stimuli during paired comparisons was ~60 to 70% of the look duration to novel stimuli.

DISCUSSION

When we designed this randomized trial in 1987, we asked if DHA was conditionally essential for optimal neural development of preterm infants. Novelty preference was chosen as one outcome. Following exposure to a stimulus (familiarization), primate infants spend more time attending to a novel stimulus (novelty preference) in a paired comparison of the familiar and novel stimuli (28). Novelty preference has been interpreted as an early measure of information processing capability (28). Under controlled conditions, novelty preference has significant, moderate predictive validity for performance on standardized intelligence tests in childhood (29,30). Novelty preference was assessed using a standardized test of novelty preference, the Fagan Test of Infant Intelligence (Fagan Test).

Shorter visual fixation or look duration in infancy has also been shown to be related to superior performance in infancy (31–33) and childhood (34). As noted earlier, short look duration has been interpreted as evidence of more efficient information processing (11,12) or enhanced ability to disengage from attended stimuli (13). At the suggestion of Dr. Susan Rose at Albert Einstein College of Medicine (personal communication), data for visual attention were also obtained. This was made possible because investigators associated with the Infantest Corporation shared software developed to measure looks and look duration during the Fagan Test. These aspects of visual attention rather than novelty preference were influenced by DHA supplementation during infancy.

Because infants were studied for fixed intervals of time at each age, more looks in DHA-supplemented compared with control infants (18) translated into significantly shorter look duration in the DHA-supplemented groups. The data reported here for look duration are, therefore, analogous to those from monkey infants (10) in that the group with better DHA status had a significantly shorter look duration during paired comparisons of novel and familiar stimuli. There was also a trend ($P < 0.12$) toward shorter look duration during familiarization in DHA-supplemented infants compared with control infants, suggesting that we would have found a similar effect during familiarization had larger numbers of infants been studied.

As shown in Table 4, there was no overall treatment (diet) effect on novelty preference with the Fagan Test, and both groups showed a significant novelty preference (the desired outcome) at all study ages. It is important to note that the Fagan Test was designed as a test of novelty preference that could screen for cognitive problems with sensitivity and identify normal cognitive function with specificity. Although effective for these purposes, the Fagan Test of novelty preference does not appear valid for identifying effects of better DHA status in normal infants.

The Fagan Test includes quite long familiarization intervals. If familiarization times are sufficiently long, they effectively ignore differences between groups in time required to habituate to a stimulus. For example, we find that preterm and term infants have a similar novelty preference on the Fagan Test (Carlson, S.E., unpublished data) even though preterm compared with term infants have not demonstrated a novelty preference in other tests with more restricted familiarization intervals (35,36). It is possible that the long familiarization times of the Fagan Test might also mask differences in novelty preference between DHA-supplemented and control infants.

We did find a lower novelty preference at 12 mon in the DHA-supplemented group compared with the group fed standard formula. This could be considered a negative finding (18); however, we do not consider this likely for several reasons. (i) The DHA-supplemented compared with the control group of infants had a similar 12-mon Bayley mental developmental index in this trial (37). (ii) The DHA-supplemented infants had a distinct novelty preference at all study ages, and their novelty preference at 12 mon did not differ from their novelty preference at 6.5 and 9 mon (the expected finding given that the Fagan Test was designed to eliminate age effects on novelty preference). (iii) Preterm infants fed DHA in this trial and a later trial (38) actually appeared to have a more mature pattern of visual attention than controls, processing information relatively more rapidly (11,12). Given long exposures in paired comparisons, infants with more mature visual information processing could habituate to the novel stimuli with a reduction in the percent of time spent viewing the novel stimulus. (iv) Little information is available about normal 12-mon novelty preference and visual behavior because studies relating these outcomes in infancy to later performance have typically involved infants between 3 and 7 mon of age.

This study confirmed that look duration decreases with age (11). We found this effect during both familiarizations and paired comparisons. Within paired comparisons, the decrease in look duration with age was seen for both novel stimuli and familiar stimuli. At each age, there was a remarkable similarity between the time/look during familiarization (when the stimulus was novel) (Table 3) and time/look to the novel stimuli during paired comparisons (Table 4).

In summary, infants fed DHA-supplemented compared with control formula until 9 mon past term had shorter look duration from 6 to 12 mon of age. Shorter look duration can now be included with higher visual acuity as a positive neural outcome of supplementing preterm infants with DHA. The relationship of DHA status to higher visual acuity and shorter look duration is analogous to those in monkey infants. Because preterm infants with the poorest DHA status received ≥3.0% linolenic acid, these data add support to earlier evidence from this randomized trial (17) that dietary DHA is conditionally essential for neural development of preterm infants.

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Visual Acuity and Erythrocyte Docosahexaenoic Acid Status in Breast-Fed and Formula-Fed Term Infants During the First Four Months of Life[1]

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ABSTRACT: It has been recognized that preterm infants have a more rapid development of visual acuity if fed human milk or a formula enriched with the long-chain polyunsaturated fatty acid (LCPUFA) docosahexaenoic acid (DHA) compared to a standard formula devoid of LCPUFA. Few studies have addressed whether the same is also true in term infants. The aim of the present study was to follow visual acuity and fatty acid composition in red blood cells (RBC) for the first 4 mon of life in 17 breast-fed and 16 formula-fed term infants. The formula used did not contain LCPUFA, but contained 1.7 wt% α-linolenic acid, and the linoleic/α-linolenic acid ratio was 8.5. The increase in visual acuity measured by Teller acuity cards developed more rapidly in breast-fed infants compared to formula-fed infants ($P < 0.001$). This was parallelled by a decrease in DHA of RBC in formula-fed infants, and with a significantly lower level at two and four months as compared to breast-fed infants. The content of DHA in milk from the breast-feeding mothers was high compared to other Western countries. The difference in visual acuity between the two feeding groups could be due to differences in DHA status as reflected by the RBC levels, but other explanations are possible. Intervention studies are required to verify if development of visual acuity in term formula-fed infants is dependent on the DHA level of formula.

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Docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6) are rapidly incorporated into brain and retina lipids during the last trimester of pregnancy and the first year of life. Human milk contains DHA and AA, which is in contrast to most formulas presently on the market for term infants (1,2). Therefore, formula-fed infants depend on endogenous synthesis, particulary of DHA, by desaturation, elongation, and β-oxidation from the precursor α-linolenic acid (18:3n-3) (3,4). The synthesis of n-3 long-chain polyunsaturated fatty acids (LCPUFA) is influenced not only by the amount of α-linolenic acid, but also by the ratio of linoleic to α-linolenic acid in the diet, because of competition between these two essential fatty acids, during the formation of LCPUFA through elongation and desaturation (5). Preterm and term infants fed a standard formula with α-linolenic acid as the only n-3 fatty acid have lower levels of plasma and red blood cell (RBC) DHA than infants fed human milk or a formula supplemented with DHA (6–10).

Data indicate that preterm infants are not able to meet their DHA needs by endogenous synthesis, i.e., DHA is conditionally essential for preterm infants. This is supported by the finding that preterm infants randomized to receive formula supplemented with n-3 LCPUFA have a more rapid visual development than preterm infants fed standard formulas devoid of LCPUFA (11,12). Few studies have compared visual acuity in term breast-fed infants and infants fed a standard formula. In two studies, visual acuity was better in the breast-fed infants (13,14), while other studies reported no difference (15,16).

The aim of the present study was to follow the development of visual acuity and the fatty acid composition of RBC during the first four months of life in term infants. The infants were fed either human milk or a formula containing no DHA, but containing 14.4. and 1.7 wt% of total fatty acids as linoleic and α-linolenic acid, respectively, giving rise to a ratio of 8.5 between these two acids. This composition resembles that of human milk and meets current recommendations (17).

MATERIALS AND METHODS

Subjects. Healthy term infants were recruited through maternity departments or health visitors during the first month after delivery. The inclusion criteria were: gestational age 37–42 wk, uncomplicated pregnancy, birth weight between 2700–4500 g, Apgar score >7 after 5 min, and no neonatal diseases. Twenty-three breast-fed and 17 formula-fed infants were included. Six breast-fed infants were excluded; two be-

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; GC, gas–liquid chromatography; LCPUFA, long-chain polyunsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; RBC, red blood cells; VEP, visual-evoked potentials.

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cause they only attended the first examination and four because the fatty acid analyses were unsuccessful.

Feeding regimen. Thus, in the breast-fed group, 17 infants were included in the analyses; one infant that received one formula meal (<150 mL/d) from three months of age was not excluded, while two infants receiving more than 500 mL of formula from the age of three months were excluded from the analyses of data from the four-month examination.

One formula-fed infant was excluded because he became ill at the age of two months. Among the remaining 16 formula-fed infants only three had never received breast milk. The median duration of breast feeding among the remaining infants was 1.5 wk (interquartile range: 3–17 d). All infants were weaned completely from the breast before the health visitor referred the mothers to us.

Small amounts of supplementary food (vegetable mashes and cereal-based gruel, not more than one meal a day) were introduced to one breast-fed and nine formula-fed infants from the age of three months.

Clinical examination and sampling of blood and milk. The infants were examined at 1, 2, and 4 mon of age. At the first visit, the parents were interviewed about pregnancy and delivery. At each visit, visual acuity, weight, length, and head circumference were measured, and a capillary blood sample was taken by a heel prick. A single milk sample (4 mL) was collected from each breast-feeding mother on all three visits, and was frozen at −80°C until analysis. Based on a review of the literature, Jensen (18) concludes that there are no differences in fatty acid composition of human milk, before and after nursing, from the right or left breast or during the day. Therefore, the mothers were asked to collect the sample at a time they found most convenient.

Fatty acid composition. RBC, obtained from 150–750 μL of blood from the infants, were subjected to lipid extraction based on the method by Folch *et al.* (19) modified for the minor amount of lipid present. Phospholipids were separated by thin-layer chromatography (20), and methyl esters were prepared from the phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fractions by saponification with 0.5 N NaOH in methanol followed by methylation of the fatty acids with 20% BF_3 in methanol as previously described (21). Methyl ester composition of PC and PE fractions from the infants was determined by gas–liquid chromatography (GC) on a Hewlett-Packard (Avondale, PA) 5890 instrument with on-column injection and a 50 m × 0.25 mm fused-silica capillary column coated with a 0.2 μm film of CP-Sil 88 (Chrompack, Middleburg, The Netherlands). The oven temperature was programmed from 90 to 160°C at a rate of 40°C/min and then to 200°C at 4°C/min, and finally, after 10 min to 220°C at a rate of 4°C/min.

Lipids from the milk were extracted (19), and methyl esters of the total lipids were prepared by direct methylation with KOH in methanol according to Christopherson and Glass (22). The fatty acid composition was determined on a Hewlett-Packard 5880A instrument with split injection and a 30 m × 0.32 mm fused-silica column with a 0.2 μm film of SP2380. The GC temperature program started from 80°C and was increased to 110°C at a rate of 30°C/min and then to 200°C at 4°C/min.

Visual acuity. Binocular acuity was measured by Teller acuity cards in half-octave steps. Infants were held by the parents and tested at 38-cm distance from the card behind a luminance matching grey screen as described by Teller *et al.* (23). The same observer (MHJ) tested all the infants at each examination. She was not blind to the feeding mode of the infants, as blinding was not possible due to practical limitations.

Statistics. Comparison between the feeding groups was done by Student's *t*-test, chi-square tests, or a two-way analysis of variance. Comparison within groups was done by using an analysis of variance. Tracking (i.e., the degree to which an infant with a high or low value at one examination adheres to a high or low value, respectively, at following examinations) was assessed using Pearson correlation coefficients.

RESULTS

There were no differences in basic characteristics (Table 1) or growth pattern (Table 2) when comparing breast-fed and formula-fed infants. Apgar scores after 5 min were ≥9 in all infants. Nor were there statistical differences between the groups with respect to mean age (SD) at the three examinations; the first examination (breast-fed vs. formula-fed) was after 24 (8.5) vs. 27 (8.7) d; the second examination after 63.5 (5.1) vs. 60 (4.8) d; and the third examination after 123 (3.1) vs. 124 (3.6) d.

Diet. The fatty acid compositions of the formula and human milk given are shown in Table 3. The formula contained only trace elements of LCPUFA and no DHA. The linoleic acid content was 14.4 wt%, slightly higher than that of human milk, and the linoleic/α-linolenic ratio was almost similar to that of the milk.

Visual acuity. Visual acuity increased over time in both feeding groups ($P < 0.001$) with a significantly higher increase in the breast-fed group ($P < 0.001$; Fig. 1). At two months, mean visual acuity was 2.6 vs. 1.8 cycles per degree (cy/degr), and at four months was 6.0 vs. 3.8 cy/degr in the breast-fed and formula-fed group, respectively.

There was no significant correlation between RBC DHA and visual acuity within the two feeding groups at four

TABLE 1
Characteristics of Infants According to Feeding Groups[a]

	Breast-fed (n = 17)	Formula-fed (n = 16)	Differences
Gestational age (wk)	40.2 (1.3)	39.8 (1.1)	n.s
Birth length (cm)	51.9 (1.9)	52.4 (2.1)	n.s
Birth weight (g)	3531 (458)	3645 (655)	n.s
Pregnancy weight gain (kg)	14.6 (4.1)	14.9 (6.4)	n.s
Maternal age (yr)	30.8 (3.8)	28.5 (3.8)	n.s
Caesarian (%)	31.3	12.5	n.s
Sex, girls (%)	50.0	25.0	n.s

[a]Values are mean (SD) or percentage; n.s., not significant.

TABLE 2
Anthropometric Data of the Two Feeding Groups[a]

		Breast-fed (n = 17)	Formula fed (n = 16)	Difference
Weight (g)	1 mon	4221.4 (587.2)	4218.9 (655.2)	n.s.
	4 mon	6827.5 (756.0)	7056.0 (976.4)	n.s.
	Weight gain (g/d)	25.9 (4.3)	28.7 (6.1)	n.s.
Length (cm)	1 mon	54.4 (1.7)	54.4 (2.7)	n.s.
	4 mon	63.9 (2.0)	64.8 (2.9)	n.s.
	Growth velocity (cm/d)	0.10 (0.02)	0.11 (0.01)	n.s.
Head circumference (cm)	1 mon	37.5 (1.1)	37.5 (1.4)	n.s.
	4 mon	42.8 (0.9)	42.6 (1.5)	n.s.
	Increase (cm/d)	0.05 (0.008)	0.05 (0.008)	n.s.

[a]Values are mean (SD); n.s., not significant.

months. However, when the two groups were combined, the correlation became significant (PC: r = 0.65, $P < 0.001$; PE: r = 0.48, $P = 0.01$).

RBC fatty acid composition. RBC DHA values were significantly higher in the breast-fed group in both the PC and PE fraction ($P < 0.001$ for both; Fig. 2). The change over time was different for the two feeding groups ($P = 0.006$ and $P < 0.001$ for PC and PE, respectively), with no change in the breast-fed group, and a significant fall in the formula-fed group for both phospholipid classes (both $P < 0.001$). At four months, the mean (SD) DHA levels in the PC fraction were 2.33 wt% (0.71) and 1.00 wt% (0.17), and in the PE fraction 8.26 wt% (2.18) and 4.20 wt% (0.63) in breast-fed and formula-fed infants, respectively.

With respect to changes over time in individual infants, the variation between infants was considerably less within the formula-fed group (Fig. 2). Tracking was also significantly higher in this group as compared with the breast-fed group. Comparing the first and the third examination for formula-fed infants, the Pearson correlation coefficient was 0.88 ($P < 0.001$) for PC and 0.67 ($P = 0.02$) for PE. For breast-fed infants there was no significant tracking.

The RBC eicosapentaenoic acid level in the PC fraction was significantly higher in the breast-fed group ($P < 0.001$), and there was no significant change over time ($P = 0.08$; Fig. 3). In the PE fraction, there was no overall difference between the feeding groups. However, the change over time was significantly different, with no change over time in the breast-fed group and a significant fall in the formula-fed group ($P = 0.009$).

In the PC fraction, RBC AA levels were significantly higher in the breast-fed group ($P < 0.001$, Fig. 3). There was a decrease over time ($P = 0.01$), mainly caused by a decrease in formula-fed infants. In the PE fraction, there was no significant difference between the feeding groups. There was no significant correlation between the AA levels and visual acuity.

Milk and formula fatty acid composition. The mean DHA in the breast milk was high at all ages (≥0.43 wt%), with no change over time (Table 3). Interestingly, two mothers devel-

TABLE 3
Fatty Acid Composition (wt%) of Milk from the Breast-Feeding Mothers and of the Formula Used[a]

	Human milk			
Fatty acid	1 mon (n = 16)	2 mon (n =17)	4 mon (n = 14)	Formula
8:0	0.18 (0.07)	0.18 (0.09)	0.15 (0.03)	
10:0	1.31 (0.44)	1.22 (0.40)	1.31 (0.85)	1.0
12:0	5.11 (1.85)	4.80 (1.83)	3.94 (1.43)	1.3
14:0	6.44 (1.64)	5.99 (1.51)	5.64 (1.89)	4.1
16:0	22.17 (1.77)	21.62 (2.52)	21.34 (2.08)	28.1
18:0	7.69 (1.05)	7.79 (1.14)	8.43 (1.80)	6.8
18:1n-9	32.23 (3.52)	32.59 (3.40)	33.96 (3.41)	35.4
18:2n-6 (LA)	10.45 (2.13)	10.97 (2.47)	11.38 (3.36)	14.4
18:n-3 (α-LA)	1.41 (0.48)	1.39 (0.47)	1.52 (0.24)	1.7
Ratio (18:2n-6/18:3n-3)	7.5	8.0	7.5	8.5
20:4n-6 (AA)	0.56 (0.12)	0.47 (0.07)	0.44 (0.09)	trace
20:5n-3 (EPA)	0.13 (0.07)	0.13 (0.04)	0.23 (0.35)	trace
22:6n-3 (DHA)	0.49 (0.20)	0.43 (0.24)	0.53 (0.56)	0

[a]Values are mean (SD); LA, linolenic acid; α-LA, α-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

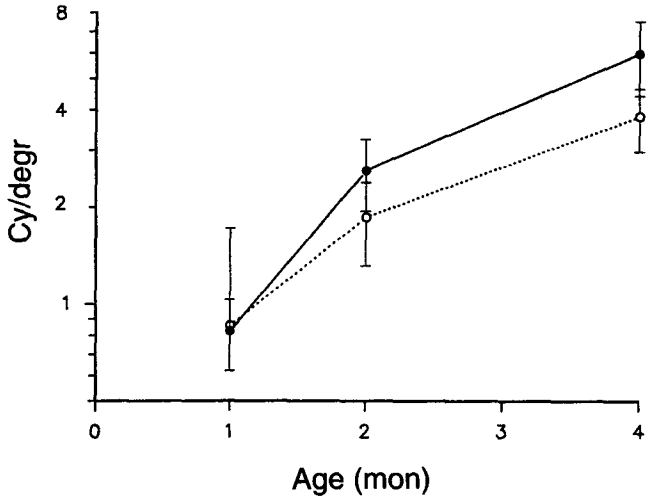


FIG. 1. Visual acuity examined by Teller cards in breast-fed and formula-fed infants. Bars are ±1.96 SEM. ●—●, Breast-fed; ○···○, formula-fed; Cy/degr, cycle per degree.

oped very high relative concentrations during the study period. We have no explanation for this increase in DHA. These two mothers reported a fish intake of 2–3 times a week thoughout the study, which was also reported in eight other cases. They did not change their fish intake, nor did they take fish oil supplements, during the study. If these high values were excluded from the analysis, there was a significant decrease in milk DHA levels from one to two months ($P = 0.01$) and a trend for a decrease from one to four months ($P = 0.09$). Furthermore, there was significant tracking from one to two months (r = 0.66, $P = 0.01$), and a trend for tracking from one to four months (r = 0.59, $P = 0.09$).

DISCUSSION

It has been argued that plasma and RBC lipids may not reflect the concentration of these fatty acids in other tissues, e.g., the central nervous system (24). However, data from infants who died by cot deaths or dehydration showed that breast-fed infants have higher levels of DHA in the cerebral cortex than infants fed a formula devoid of DHA (25,26). Furthermore, it was shown that the level of DHA in cerebral cortex increases with duration of breast-feeding in contrast to formula-fed infants (26). However, no such difference was observed in the retina.

Because the concentration of DHA is particularly high in the synaptosomes of the central nervous system and the outer rod segment of the retina, it is to be expected that symptoms of deficiency should first appear from these tissues. Indeed, the first deficiency symptom in the human species reported was a decrease in visual function. In two randomized studies, preterm infants fed a formula supplemented with fish oil, providing DHA, was found to have a more rapid development of visual acuity as assessed by Teller cards (12) and visual evoked potentials (VEP) (11) than infants fed a controlled formula without DHA.

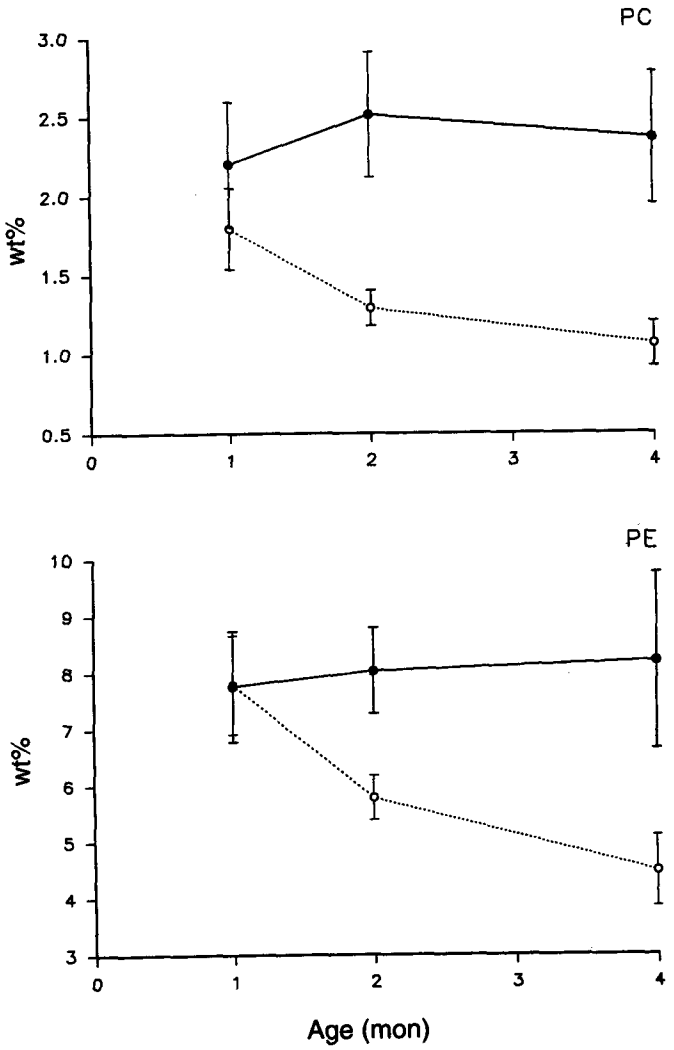


FIG. 2. Red blood cell phospholipid relative docosahexaenoic acid concentration in breast-fed and in formula-fed infants. Bars are ±1.96 SEM; ●—●, breast-fed; ○···○, formula-fed; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

Makrides *et al.* (13), in a nonrandomized study, were the first to report a difference in visual acuity measured by VEP between breast-fed term infants and term infants fed a formula without DHA. On the other hand, Innis *et al.* (15) suggested that when term infants are fed a formula containing 2.1 wt% of the fatty acids as α-linolenic acid, there is no difference in visual acuity measured by Teller cards at three months of age between formula-fed and breast-fed infants. The formula used in the present study contained 1.7 wt% of the fatty acids as α-linolenic acid, which is close to the level used by Innis *et al.* (15), and with the same linoleic to α-linolenic acid ratio. In contrast to Innis *et al.* (27) and others (16), we found a highly significant difference in visual acuity at both two and four months of age (28).

The levels of linoleic and α-linolenic acids were chosen to resemble those of human milk from Danish mothers and to meet current recommendations of the European Society for Pediatric Gastroenterology and Nutrition (17). As the

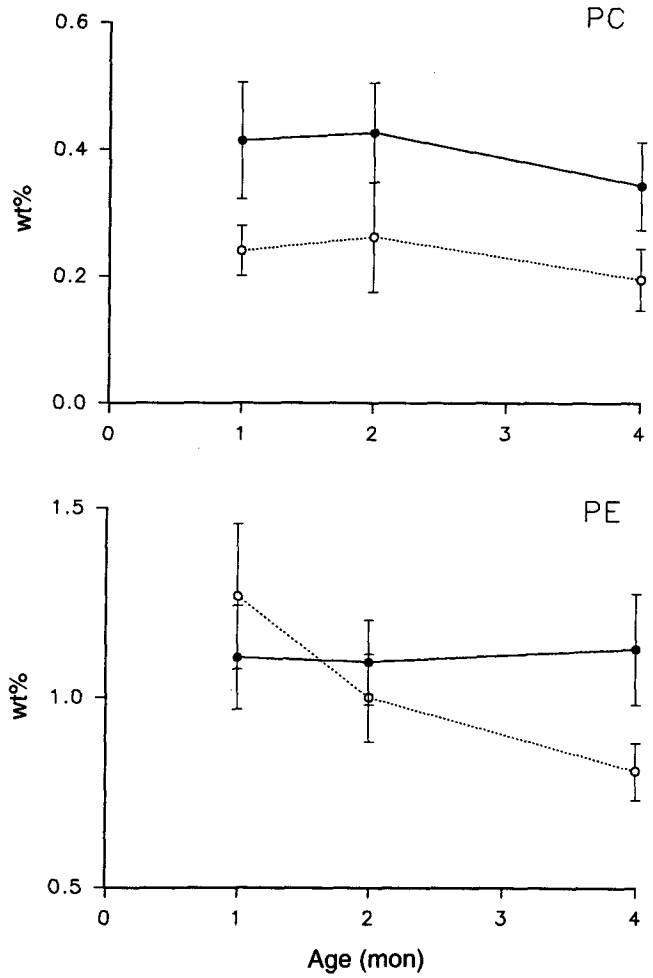


FIG. 3. Red blood cell phospholipid relative eicosapentaenoic acid concentration in breast-fed and in formula-fed infants. Bars are ±1.96 SEM; ●—●, breast-fed; ○···○, formula-fed; abbreviations as in Figure 2.

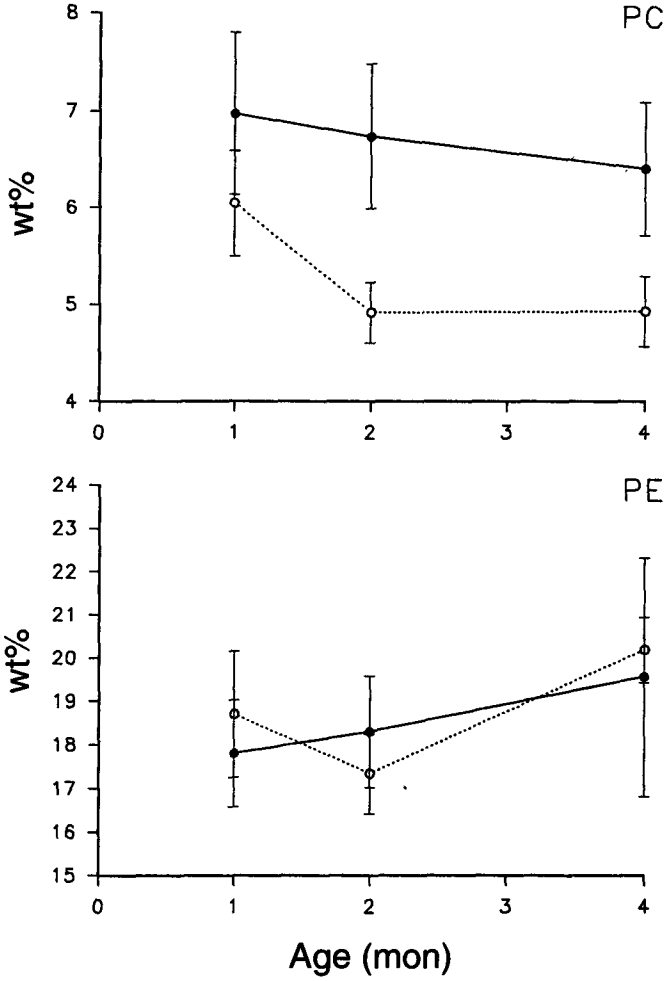


FIG. 4. Red blood cell phospholipid relative arachidonic acid concentration in breast-fed and in formula-fed infants. Bars are ±1.96 SEM; ●—●, breast-fed; ○···○, formula-fed; abbreviations as in Figure 2.

linoleic/α-linolenic fatty acid ratio is very similar to that used in the previously mentioned studies, it seems unlikely that differences in composition of the formulas could explain the observed differences with respect to visual acuity.

The hypothesis that the difference in DHA intake explains the difference in visual acuity in our term infants is supported in a recent study by Makrides *et al.* (29). They randomized term infants to feeding with a formula without DHA or one with 0.36 wt% DHA and compared with a breast-fed group. They found a higher visual acuity, measured by VEP in the breast-fed and in infants receiving the supplemented formulas, than in infants receiving the unsupplemented formula at 15 and 30 wk of age.

In agreement with others, we found that the relative concentration of RBC DHA decreased in formula-fed infants over time (9). This was true both for the PC and PE fractions of RBC. In contrast, in breast-fed infants, there was no significant decrease in DHA during the first four months of age. The main part of RBC AA was found within the PE fraction. We found no significant difference between the feeding groups in this fraction, although breast-fed infants had significantly higher values in the PC fraction.

In the study of Innis *et al.* (15), the relative concentrations of PC DHA, but not PE DHA, were lower both in breast-fed and in formula-fed infants compared to our study. We cannot explain this, nor do we know if this relates to differences in outcome. It is interesting to note, however, that we found considerably higher levels of DHA in breast milk than did Innis *et al.* (15). The medium levels in the present study, excluding the two mothers with the very high levels, were 0.49, 0.40, and 0.32 wt% at 1, 2, and 4 mon, respectively. In another study including Danish mothers, we found a level of 0.33 wt% (2). This is higher than the 0.2 wt% in the study by Innis *et al.* (15), and values reported from the United States of America, Australia, Germany, Hungary, and Sweden (29–32). This could explain the higher relative concentration in RBC phospholipids in our breast-fed infants.

We found a strong positive association between RBC DHA and visual acuity, which is in accordance with Makrides *et al.* (13). However, it is well known that DHA levels are different

in breast-fed and in formula-fed infants, and the positive association found when combining the two groups is therefore not a strong support that DHA status is important for visual acuity. When we analyzed the association between RBC DHA and visual acuity within each of the two groups separately, the associations were not significant. This could be due to the relatively low number of infants in the groups. In a randomized intervention study of term infants, Carlson *et al.* (33) found a difference in visual acuity at two months but not at four months of age when comparing two different formulas: one with a DHA level of 0.1 wt%, and the other being a standard formula without DHA. Thus, a small amount of DHA in the formula was associated with a transcient improvement in visual acuity, supporting a role for DHA. Furthermore, in the study by Makrides *et al.* (13), DHA was the only fatty acid that correlated to VEP acuity. Hence, it is tempting to speculate that the discrepancy with respect to effect on visual acuity in studies comparing breast-fed and formula-fed term infants could be explained by differences in DHA intake.

Finally, it should be noted that compositional differences between breast milk and formula other than the level of DHA or other polyunsaturated fatty acids might affect the development of visual acuity, compounds that are as yet unknown. The observer was not blinded to the feeding mode when testing visual acuity. Therefore we cannot exclude that the difference found was caused by observed bias. Furthermore, breast feeding *per se* might contribute to the outcome: one reason being that mothers who choose to breast feed may spend more time with their babies and by various means promote their development, including visual function.

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Biochemical Effects of Dietary Linoleic/α-Linolenic Acid Ratio in Term Infants[1]

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ABSTRACT: Recent statements concerning linoleic (LA) and α-linolenic acid (LNA) intakes for infants include a desirable range of LA/LNA ratios. To evaluate several dietary LA/LNA ratios, the fatty acid patterns of plasma and erythrocyte phospholipid fractions, as well as plasma total lipid fractions, were determined shortly after birth and at 21, 60, and 120 d of age in term infants fed formula with 16% of fat as LA and either 0.4, 0.95, 1.7, or 3.2% as LNA (LA/LNA ratios of approximately 44, 18, 10, and 5). The content of all n-3 fatty acids in both plasma fractions was higher at all times in infants who received the highest LNA intake; however, the docosahexaenoic acid (DHA) content was only half that shortly after birth or reported in breast-fed infants of comparable ages. The LA content of plasma lipids of all groups was higher at all times than shortly after birth but did not differ among groups. The arachidonic acid (AA) content was higher in infants who received the lowest LNA intake, but only half that at birth or reported in breast-fed infants. In contrast, the DHA content of the erythrocyte phospholipid fraction did not differ among groups until 120 d of age when it was higher in those who received the highest LNA intake and the AA content of this fraction did not differ among groups at any time. These data demonstrate that dietary LA/LNA ratios between 5 and 44 do not result in plasma or erythrocyte lipid levels of DHA or plasma lipid levels of AA similar to those at birth or reported by others in breast-fed infants. However, the data indicate that the LA/LNA ratio of the formula is an important determinant of the amounts of DHA and AA required to achieve plasma and erythrocyte levels of these fatty acids similar to those of breast-fed infants.

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Docosahexaenoic acid (22:6n-3; DHA) and arachidonic acid (20:4n-6; AA) are important constituents of the structural lipids of membranes, particularly those of the retina and brain (1–4). Although both fatty acids are present in human milk, neither is present in infant formulas currently marketed in the United States. Consequently, the formula-fed infant must synthesize these fatty acids from their 18-carbon precursors, α-linolenic (18:3n-3; LNA) and linoleic acid (18:2n-6; LA), respectively. However, there is considerable evidence that formula-fed infants cannot synthesize sufficient DHA and AA to maintain plasma and erythrocyte levels of these fatty acids comparable to those of breast-fed infants (5–11). Recent postmortem studies (12–14) show that the brain content of DHA, but not AA, also is lower in formula-fed vs. breast-fed infants.

Since infants can synthesize some DHA and AA (15–17), the amounts of dietary LNA and LA, which compete with each other for desaturation and elongation, would be expected to affect endogenous DHA and AA synthesis. However, although a number of national and international advisory groups have recommended specific ranges of intakes of LNA and LA, including the ratio of the two (18–22), data concerning the effects of different dietary LA and LNA intakes and/or different dietary LA/LNA ratios on plasma and/or tissue DHA and AA levels of infants are limited. While ratios as low as four have been recommended (23), only one study, to our knowledge, has included infants fed a dietary LA/LNA ratio lower than seven (7).

The study reported here was designed to determine the effects of four LNA intakes, all accompanied by the same moderate intake of LA and, hence, the effects of four dietary ratios of LA/LNA on fatty acid patterns of plasma and erythrocyte lipid fractions of term infants. The data suggest that the LA/LNA ratio is an important determinant of the amount of DHA and/or AA that must be added to formulas to achieve the same plasma and/or erythrocyte lipid DHA and AA levels observed in breast-fed infants.

METHODS

Study population and study design. Eighty healthy term infants born after at least 37 weeks' gestation (mean gestational age, 39.6 ± 1.7 wk) with birth weights between the 5th and 95th percentiles of the National Center for Health Statistics standards (24) and whose mothers had elected not to breast-feed were assigned randomly and blindly to receive formulas

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; LNA, α-linolenic acid.

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that differed only in LNA content as their sole source of nutrition from birth to 120 d of age. The fatty acid composition of the formulas and the oils comprising their fat content are shown in Table 1. LA comprised 15.6–17.6% of the total fatty acids of all formulas; LNA content varied from 0.4% in Formula 1 to 3.24% in Formula 4. The LA/LNA ratios of the formulas, which were manufactured by Mead Johnson Nutritionals (Evansville, IN), were 44.0, 18.2, 9.7, and 4.8; their total fat, protein, energy, carbohydrate, vitamin, and mineral contents were similar to those of the standard infant formula, Enfamil (Mead Johnson Nutritionals).

The assigned formula was started during the first day of life, and the intake of each infant throughout the first 120 d of life was estimated as described below. The fatty acid composition of the plasma total lipid fraction as well as the plasma and erythrocyte phospholipid fractions was measured within two days of birth (prior to hospital discharge) and within five days of 21, 60, and 120 d of age (see below).

The study was approved by the Clinical Investigation Committees of Baylor College of Medicine, Texas Children's Hospital, and Harris County Hospital District. Signed informed consent was obtained from at least one parent of all infants enrolled.

Formula intake. Intake of formula during the brief period of hospitalization was determined as the difference between pre- and postfeeding weights of each feeding unit offered. At discharge, at least one case of preweighed ready-to-feed formula was given to the parents at discharge, and additional preweighed cases were delivered to their homes as necessary thereafter (usually weekly or every other week). At each formula delivery, unused formula from the previous delivery was retrieved and weighed. Formula intake during the interval between deliveries was calculated as the difference between the weight of the case of formula delivered and that of the retrieved case. To facilitate calculation of intake during specific intervals (e.g., discharge to 21 d; 21 to 60 d, etc.), a fresh preweighed case of formula was always started immediately after each post-discharge evaluation.

Parents were instructed not to discard any unused formula but, rather, to replace the original lid on each bottle and return it to the case. Inspection of the returned cases indicated excellent compliance with this instruction.

Plasma and erythrocyte fatty acid patterns. Prior to hospital discharge and within five days of 21, 60, and 120 d of age, blood was obtained in EDTA-containing tubes for determining the fatty acid pattern of plasma and erythrocyte lipid fractions. Plasma and erythrocytes were separated immediately by centrifugation and stored separately at −70°C until analysis. For analysis, plasma and erythrocyte lipids were extracted by the method of Bligh and Dyer (25), and the phospholipid fractions of each were separated by one-dimensional thin-layer chromatography (Silica Gel G) using hexane/diethyl ether/glacial acetic acid (70:35:1, by vol). Methyl esters of the component fatty acids of the plasma total lipid and phospholipid fractions, as well as the erythrocyte phospholipid fraction, were prepared with boron trifluoride–methanol (26) and quantitated by gas–liquid chromatography (Varian 3500, Walnut Creek, CA) on a DB-225 capillary column (J&W Scientific, Folsom, CA) in the presence of 17:0 and 21:0 internal standards (27). The fatty acid patterns of the extracted lipid of the four formulas were determined by the same methods (Table 1).

Data analysis. All data are expressed as group means ± SD. Statistical significance of differences in means among groups at each time and differences within and/or among groups over time was tested by analysis of variance or covariance for repeated measures. The value of each fatty acid variable prior to hospital discharge was used as the covariate. Analyses of variance were followed, if indicated, with the Fisher least significance difference procedure for multiple comparisons of each paired group difference. A probability of 5% was assumed to represent statistical significance.

TABLE 1
Fatty Acid Composition (weight percent total fatty acids) of Study Formulas[a]

Fatty acid	Formula 1	Formula 2	Formula 3	Formula 4
8:0	1.3	1.2	1.3	1.2
10:0	0.9	0.9	1.0	1.0
12:0	9.7	9.6	9.6	9.5
14:0	4.7	4.6	4.6	4.6
16:0	23.6	23.3	23.4	23.2
18:0	4.5	4.5	4.5	3.7
20:0	0.3	0.3	0.3	0.3
16:1n-7	0.1	0.15	0.12	0.2
18:1n-9	36.5	36.9	36.6	36.6
22:1n-11	0.02	0.04	0.07	0.3
18:2n-6	17.6	17.3	16.5	15.6
18:3n-3	0.4	0.95	1.7	3.24
18:2n-6/18:3n-3	44.0	18.2	9.7	4.8

[a]The fat content of the formulas included canola, safflower, high-oleic sunflower, palm–stearin, and coconut oils: Formula 1, 3% canola, 15% safflower, 26% high-oleic sunflower, 30% palm stearin, and 26% coconut; Formula 2, 10% canola, 13% safflower, 22% high-oleic sunflower, 30% palm stearin, and 25% coconut; Formula 3, 20% canola, 10% safflower, 15% high-oleic sunflower, 30% palm stearin, and 25% coconut; Formula 4, 40% canola, 5% safflower, 30% palm stearin, and 25% coconut.

RESULTS

Subjects. Sixty-three of the eighty infants enrolled completed the study. Reasons for noncompletion included paternal disapproval (n = 4), unexplained decision to terminate participation (n = 4), maternal desire to attempt breast feeding (n = 2), dietary protein hypersensitivity (n = 2), and change of address (n = 5). The general characteristics of the four completed groups did not differ. Mean birth weights (kg ± SD) of Groups 1 through 4, respectively, were 3.38 ± 0.35 (n = 17), 3.20 ± 0.39 (n = 17), 3.15 ± 0.50, (n = 16), and 3.25 ± 0.48 (n = 13). Mean gestational ages (wk ± SD), respectively, were 39.8 ± 1.4, 39.6 ± 1.8, 39.6 ± 2.0, and 39.6 ± 1.5. Groups 1 and 2 included seven females and ten males; the female/male distribution of Groups 3 and 4 were 10:6 and 7:6. The Black/Hispanic/Caucasian distribution of the four completed groups

were: Group 1, 13:2:2; Group 2, 13:2:2; Group 3, 8:7:1; Group 4, 5:7:1.

The three postdischarge studies were performed at mean ages of 23.5 ± 4.5, 62.0 ± 5.3, and 124.4 ± 3.4 d in Group 1; 21.9 ± 1.5, 62.3 ± 4.7, and 123.4 ± 5.4 d in Group 2; 22.9 ± 3.1, 62.1 ± 2.1, and 122.2 ± 2.4 d in Group 3; and 22.0 ± 3.3, 60.2 ± 2.4, and 119.7 ± 3.1 d in Group 4.

Formula intake. There were no statistically significant differences in formula intake among groups either before hospital discharge or during any of the study intervals (data not shown). By history, none of the infants received foods other than the assigned formula before 120 d of age.

Plasma and erythrocyte fatty acid patterns. The mean mole percent of saturated and monounsaturated as well as individual n-3 and n-6 polyunsaturated fatty acids in the plasma phospholipids of all groups shortly after birth and at each postdischarge study are shown in Table 2. There were few statistically significant differences in the plasma phospholipid fatty acid patterns of individual groups at 21 vs. 60 vs. 120 d of age (i.e., group differences were apparent by 21 d of age and persisted through 120 d of age). However, the percentage of many fatty acids at these times differed from the percentage shortly after birth.

There were no statistically significant differences in the plasma phospholipid content of saturated or monounsaturated fatty acids among the formula groups at any time. At all times postdischarge, the percentages of 18:3n-3 (LNA), 20:5n-3, and 22:5n-3 were highest in the group that received the highest LNA intake (Group 4) and lowest in the group that received the lowest LNA intake (Group 1). The DHA (22:6n-3) content was lower than shortly after birth in all groups at all times postdischarge, but the content of Group 4 was significantly higher than that of the other three groups, the contents of which did not differ significantly.

The 18:2n-6 (LA) content of the plasma phospholipid fraction was higher than shortly after birth in all groups at 21, 60, and 120 d but did not differ among groups. The percentage of 20:4n-6 (AA) was significantly lower in all groups at 21, 60, and 120 d than shortly after birth, but, at all times, the percentage was higher in Group 1, which received the lowest LNA intake, than in Groups 2, 3, and 4. The percentages of both 22:4 n-6 and 22:5 n-6 were lowest at all times postdischarge in Group 4, which received the highest LNA intake, and highest in Group 1, which received the lowest LNA intake. The percentages of these fatty acids in Groups 2 and 3 were intermediate.

The mole percentages of most fatty acids in the plasma total lipid fraction (data not shown) differed quantitatively from the percentages in the plasma phospholipid fraction but the relative differences among groups and over time were similar to those observed in the plasma phospholipid fraction. Thus, these data and the fatty acid concentrations of the plasma phospholipid and total lipid fraction, which also did not provide additional information, are not shown (they can be obtained upon request from the authors).

The mean mole percent of total saturated and monounsaturated as well as selected n-3 and n-6 fatty acids of the erythrocyte phospholipid fractions of all groups at each time are shown in Table 3. For the most part, the fatty acid patterns of this fraction reflected the same relative differences among

TABLE 2
Fatty Acid Pattern of Plasma Phospholipid Fraction (mole percent) (from birth to 120 d of age)[a]

	Birth	21 Days	60 Days	120 Days
Total saturated fatty acids				
Group 1	45.3 ± 0.9	43.9 ± 0.7	44.7 ± 0.9	44.8 ± 0.7
Group 2	45.5 ± 0.9	44.2 ± 0.6	44.5 ± 0.8	44.8 ± 0.8
Group 3	45.3 ± 0.7	43.8 ± 0.6	44.6 ± 0.9	44.5 ± 0.8
Group 4	45.0 ± 0.9	43.9 ± 0.5	44.5 ± 0.8	44.3 ± 0.9
Total monounsaturated fatty acids				
Group 1	15.1 ± 1.0	14.9 ± 0.7	12.9 ± 1.9	12.4 ± 1.5
Group 2	15.6 ± 1.2	15.1 ± 0.7	13.7 ± 0.6	12.2 ± 1.4
Group 3	15.4 ± 1.4	15.7 ± 1.0	14.1 ± 0.8	13.3 ± 1.6
Group 4	15.1 ± 1.2	15.5 ± 0.7	13.9 ± 1.5	13.4 ± 1.9
18:3n-3				
Group 1	0.10 ± 0.05	0.04 ± 0.05[a]	0.11 ± 0.13[a]	0.09 ± 0.17[a]
Group 2	0.09 ± 0.04	0.11 ± 0.04[b]	0.11 ± 0.02[a]	0.16 ± 0.10[a,b]
Group 3	0.11 ± 0.05	0.17 ± 0.07[c]	0.19 ± 0.06[b]	0.21 ± 0.08[b]
Group 4	0.20 ± 0.10	0.35 ± 0.07[d]	0.39 ± 0.06[c]	0.48 ± 0.09[c]
20:5n-3				
Group 1	0.11 ± 0.05	0.03 ± 0.04[a]	0.06 ± 0.08[a]	0.11 ± 0.08[a]
Group 2	0.10 ± 0.07	0.10 ± 004[b]	0.09 ± 0.04[a]	0.11 ± 0.06[a]
Group 3	0.12 ± 0.04	0.18 ± 0.08[c]	0.16 ± 0.06[b]	0.15 ± 0.08[b]
Group 4	0.13 ± 0.09	0.31 ± 0.11[d]	0.31 ± 0.10[c]	0.29 ± 0.11[c]
22:5n-3				
Group 1	0.21 ± 0.05	0.13 ± 0.05[a]	0.27 ± 0.20[a]	0.31 ± 0.25[a]
Group 2	0.20 ± 0.07	0.24 ± 0.07[b]	0.29 ± 0.07[a]	0.37 ± 0.15[a,b]
Group 3	0.19 ± 0.06	0.35 ± 0.07[c]	0.45 ± 0.10[b]	0.47 ± 0.15[b]
Group 4	0.22 ± 0.07	0.46 ± 0.07[d]	0.57 ± 0.13[c]	0.62 ± 0.15[c]
22:6n-3				
Group 1	3.94 ± 0.89	1.59 ± 0.47[a]	1.38 ± 0.61[a]	1.13 ± 0.41[a]
Group 2	3.41 ± 0.95	1.55 ± 0.41[a]	1.32 ± 0.33[a]	1.21 ± 0.38[a]
Group 3	3.36 ± 0.97	1.83 ± 0.35[a]	1.66 ± 0.30[a]	1.27 ± 0.35[a]
Group 4	3.41 ± 0.80	2.25 ± 0.57[b]	2.25 ± 0.66[b]	1.72 ± 0.66[b]
18:2n-6				
Group 1	16.7 ± 3.7	27.5 ± 1.8	28.9 ± 2.6	30.2 ± 1.8
Group 2	16.3 ± 4.1	28.2 ± 1.3	29.6 ± 2.2	31.2 ± 2.3
Group 3	17.0 ± 3.2	27.7 ± 1.2	28.7 ± 2.4	31.1 ± 1.7
Group 4	16.7 ± 3.2	27.5 ± 1.7	29.1 ± 2.5	30.9 ± 2.6
20:4n-6				
Group 1	15.0 ± 2.4	8.0 ± 1.4[a]	8.1 ± 2.0[a]	7.6 ± 1.6[a]
Group 2	15.4 ± 2.9	7.0 ± 1.0[b]	6.9 ± 1.4[a,b]	6.9 ± 1.5[a,b]
Group 3	14.7 ± 2.7	6.6 ± 0.8[b]	6.6 ± 1.3[b]	6.2 ± 1.4[b]
Group 4	15.2 ± 2.4	6.4 ± 1.5[b]	6.2 ± 2.0[b]	5.8 ± 1.9[b]
22:4n-6				
Group 1	0.48 ± 0.13	0.39 ± 0.06[a]	0.46 ± 0.11[a]	0.48 ± 0.09[a]
Group 2	0.49 ± 0.15	0.33 ± 0.05[b]	0.41 ± 0.07[a]	0.42 ± 0.07[a]
Group 3	0.49 ± 0.13	0.30 ± 0.04[b,c]	0.34 ± 0.07[b]	0.36 ± 0.09[b]
Group 4	0.50 ± 0.12	0.26 ± 0.08[c]	0.26 ± 0.07[c]	0.27 ± 0.09[c]
22:5n-6				
Group 1	0.89 ± 0.3	0.81 ± 0.21[a]	0.81 ± 0.38[a]	0.64 ± 0.23[a]
Group 2	0.95 ± 0.3	0.63 ± 0.20[b]	0.65 ± 0.15[a,b]	0.52 ± 0.17[b]
Group 3	0.89 ± 0.3	0.50 ± 0.13[c]	0.51 ± 0.19[b,c]	0.40 ± 0.16[b]
Group 4	0.88 ± 0.3	0.36 ± 0.11[d]	0.33 ± 0.09[c]	0.27 ± 0.06[c]

[a]Statistically significant differences ($P < 0.05$) among groups at each time are indicated by different superscripts (a–d).

TABLE 3
Fatty Acid Pattern of Erythrocyte Phospholipid Fraction (mole percent) (from birth to 120 d of age)[a]

	Birth	21 Days	60 Days	120 Days
Total saturated fatty acids				
Group 1	53.6 ± 8.8	44.4 ± 4.1	43.0 ± 3.5	41.1 ± 0.5
Group 2	50.5 ± 5.8	44.1 ± 3.2	42.4 ± 3.0	42.6 ± 6.4
Group 3	51.4 ± 8.3	42.0 ± 0.9	43.2 ± 5.3	40.8 ± 0.8
Group 4	51.5 ± 8.2	43.7 ± 3.0	41.7 ± 0.8	40.8 ± 0.6
Total monounsaturated fatty acids				
Group 1	21.6 ± 2.8	19.2 ± 2.4	18.8 ± 2.2	17.7 ± 1.4
Group 2	20.8 ± 3.4	19.7 ± 2.3	19.1 ± 1.6	18.5 ± 2.1
Group 3	20.8 ± 3.9	18.6 ± 1.6	19.7 ± 1.5	19.1 ± 1.3
Group 4	20.2 ± 3.7	19.6 ± 2.1	19.4 ± 1.5	18.9 ± 1.8
18:3n-3				
Group 1	0.03 ± 0.05[a,b]	0.01 ± 0.03[a]	0.04 ± 0.07[a]	0.05 ± 0.11[a]
Group 2	0.01 ± 0.03[b]	0.06 ± 0.05[b]	0.07 ± 0.05[a,b]	0.09 ± 0.06[a,b]
Group 3	0.01 ± 0.04[b]	0.10 ± 0.04[c]	0.09 ± 0.05[b]	0.11 ± 0.06[b]
Group 4	0.05 ± 0.07[a]	0.18 ± 0.07[d]	0.23 ± 0.07[c]	0.23 ± 0.05[c]
20:5n-3				
Group 1	0.03 ± 0.05	0.04 ± 0.05[a]	0.04 ± 0.06[a]	0.11 ± 0.10[a]
Group 2	0.05 ± 0.05	0.09 ± 0.10[b]	0.10 ± 0.00[b]	0.11 ± 0.05[a]
Group 3	0.03 ± 0.05	0.10 ± 0.04[b]	0.12 ± 0.06[b]	0.17 ± 0.07[b]
Group 4	0.05 ± 0.05	0.19 ± 0.06[c]	0.30 ± 0.06[c]	0.34 ± 0.05[c]
22:5n-3				
Group 1	0.25 ± 0.13	0.34 ± 0.09[a]	0.51 ± 0.21[a]	0.80 ± 0.59[a]
Group 2	0.28 ± 0.14	0.42 ± 0.09[a,b]	0.69 ± 0.13[b]	0.94 ± 0.32[a]
Group 3	0.27 ± 0.16	0.53 ± 0.10[b]	0.93 ± 0.20[c]	1.30 ± 0.26[b]
Group 4	0.36 ± 0.22	0.69 ± 0.23[c]	1.45 ± 0.22[d]	1.94 ± 0.24[c]
22:6 n-3				
Group 1	2.79 ± 1.73	3.48 ± 1.18	2.35 ± 0.79	1.63 ± 0.35[a]
Group 2	3.25 ± 1.91	3.18 ± 1.47	2.27 ± 0.72	1.50 ± 0.52[a]
Group 3	3.11 ± 2.03	3.81 ± 0.98	2.47 ± 0.70	1.73 ± 0.51[a]
Group 4	3.20 ± 2.10	3.39 ± 1.23	3.13 ± 0.74	2.39 ± 0.76[b]
18:2n-6				
Group 1	4.43 ± 1.08	11.14 ± 1.89	12.52 ± 2.04	13.25 ± 1.24
Group 2	4.73 ± 0.80	11.51 ± 1.67	12.64 ± 1.63	12.95 ± 2.51
Group 3	5.02 ± 1.38	12.58 ± 1.51	12.51 ± 2.45	13.11 ± 1.53
Group 4	4.93 ± 1.26	11.37 ± 1.62	12.31 ± 1.73	13.11 ± 1.52
20:4n-6				
Group 1	12.1 ± 6.1	14.6 ± 2.9	15.7 ± 3.0	18.1 ± 1.8
Group 2	14.2 ± 4.6	14.4 ± 2.0	15.7 ± 2.3	16.7 ± 3.9
Group 3	13.4 ± 6.3	15.1 ± 1.2	14.7 ± 2.9	17.5 ± 1.5
Group 4	13.4 ± 5.8	14.3 ± 2.0	15.1 ± 1.1	16.6 ± 1.5
22:4n-6				
Group 1	2.47 ± 1.34	3.29 ± 0.91	3.75 ± 0.94[a]	4.17 ± 0.59[a]
Group 2	2.85 ± 1.07	3.13 ± 0.73	3.58 ± 0.68[a,b]	3.69 ± 1.03[a,b]
Group 3	2.62 ± 1.23	3.47 ± 0.58	3.20 ± 0.79[b,c]	3.45 ± 0.42[b]
Group 4	2.82 ± 1.45	3.13 ± 0.79	3.20 ± 0.28[c]	2.93 ± 0.34[c]
22:5n-6				
Group 1	1.31 ± 0.84	1.71 ± 0.67	1.53 ± 0.49[a]	1.31 ± 0.38[a]
Group 2	1.63 ± 0.68	1.59 ± 0.60	1.45 ± 0.42[a]	1.07 ± 0.36[b]
Group 3	1.43 ± 0.73	1.71 ± 0.36	1.15 ± 0.29[b]	0.89 ± 0.24[b,c]
Group 4	1.41 ± 0.83	1.41 ± 0.57	1.15 ± 0.34[b]	0.70 ± 0.15[c]

[a]Statistically significant differences ($P < 0.05$) among groups at each time are indicated by different superscripts (a–d).

groups as the plasma phospholipid fraction. However, there were some important differences. The percentage of DHA in erythrocyte phospholipids at 21 and 60 d did not differ significantly from that shortly after birth in any group, and there were no statistically significant differences among groups; however, at 120 d, the percentage of DHA was lower in all groups than shortly after birth and significantly lower in Groups 1, 2, and 3 than in Group 4, which received the highest LNA intake. There were no statistically significant differences in the AA content of this fraction among groups at any time, and in all groups, the percentage at all times was from 18% (Group 2) to 50% (Group 1) higher than shortly after birth. The percentage of 22:4n-6 and 22:5n-6 did not differ among groups at 21 d but thereafter, the percentage of these fatty acids was lower, although not necessarily lower than shortly after birth, in Group 4 vs. Groups 1, 2, and 3.

DISCUSSION

As reported above and as expected, the highest LNA intake studied, or the lowest LA/LNA ratio resulted in higher plasma phospholipid and total lipid levels of DHA; the lowest LNA intake studied, or the highest ratio, resulted in higher AA levels. However, the DHA and AA contents of these plasma lipid fractions from 21 to 120 d of age were lower than shortly after birth and also lower than reported by others in breast-fed infants (8). Thus, no dietary LA/LNA ratio between 4.8 and 44 results in plasma lipid levels of either DHA or AA similar to those of breast-fed infants. Moreover, examination of the plasma phospholipid content of all n-3 and n-6 fatty acids suggests that this goal is unlikely to be achieved with either a lower or a higher dietary LA/LNA ratio.

The highest vs. the lowest LNA intake resulted in a severalfold greater plasma phospholipid content of LNA and EPA (20:5n-3), but only a modestly greater content of DHA. Similarly, this LNA intake resulted in a modestly lower plasma phospholipid content of AA but a markedly lower content of 22:5n-6. Similar but less marked effects also were observed by Clark *et al.* (7) in term infants fed formulas with 3.3 vs. 0.7% of fat as LNA (both with 13–14% of fat as LA). Ponder *et al.* (8) observed a higher eicosapentaenoic acid (EPA) content of plasma phospholipids in term infants fed formulas with 4.8 vs. 0.8% of fat as LNA (with 34 and 31% of fat, respectively, as LA) but no difference in the content of LNA, AA, or 22:5n-6. Marked increases in the LNA, EPA, and 22:5n-3 contents of the plasma phospholipid fraction and an actual decrease in DHA content were observed by Anderson *et al.* (28) in dogs that received a much higher intake of LNA and by Mantzioris *et al.* (29) in human volunteers who received 5.3% of energy intake as LNA and 3.3% as LA. The effect of the high LNA intake on n-6 fatty acids observed by Anderson *et al.* (28) in dogs and Mantzioris *et al.* (29) in human volunteers also was similar to the effect reported here, i.e., a modest decrease in plasma phospholipid AA but a more dramatic decrease in plasma phospholipid 22:4n-6 and 22:5n-6.

These effects of LNA intake on the content of long-chain, polyunsaturated n-3 and n-6 fatty acids in plasma lipids are difficult to explain totally by competition between LNA and LA for Δ^6-desaturation, respectively, to 18:4n-3 and 18:3n-6.

Mantzioris *et al.* (29) speculated that the inverse relationship between dietary LNA and the DHA content of phospholipids might represent displacement of DHA by EPA or by LNA itself. The effect also can be explained by competition of LNA, and possibly LA, with 24:5n-3 and 24:4n-6 for Δ^6-desaturation, respectively, to 24:6n-3 to 24:5n-6. These 24-carbon fatty acids are part of a pathway that was described recently in isolated hepatocytes (30,31) and has been shown to be involved in *in vivo* synthesis of 22:6n-3 by human infants (32,33). In this pathway, 22:5n-3 and 22:4n-6, rather than, and/or in addition to, undergoing Δ^4-desaturation to 22:6n-3 and 22:5n-6, respectively, are elongated to 24:5n-3 and 24:4n-6, which then undergo Δ^6-desaturation, and the products, 24:6n-3 and 24:5n-6, in turn, undergo β-oxidation, presumably in peroxisomes, to 22:6n-3 and 22:5n-6. Alternatively, or additionally, increased 22:5n-3 incident to the higher LNA intake could compete with 22:4n-6 for Δ^4-desaturation of 22:5n-3 to 22:6n-3 and 22:4n-6 to 22:5n-6. However, a Δ^4-desaturase has never been detected, and some believe that there may be no such enzyme (34,35).

Whatever the specific mechanism, the implications of the observed effects of LNA intake on n-3 and n-6 fatty acid metabolism are clear. If LNA, the precursor of DHA, interferes directly with synthesis of DHA as well as the corresponding n-6 polyunsaturated fatty acid (22:5n-6), an intake of LNA greater than the highest intake studied here, or a LA/LNA ratio lower than the lowest ratio studied, might reduce rather than increase the plasma phospholipid content of DHA, as observed by Anderson *et al.* (28) in dogs and Mantzioris *et al.* (29) in human volunteers.

Since the dietary LA/LNA ratio affects the plasma phospholipid content of both DHA and AA, it is clear that the intake of DHA and/or AA required to produce plasma lipid levels of these fatty acids similar to those of breast-fed infants will depend upon the LA/LNA ratio of the formula to which they are added. In other words, current recommendations to supplement infant formulas with the amounts of DHA and AA in human milk may only be appropriate for formulas with the same LA and LNA contents as human milk. Unfortunately, the LA and LNA, as well as the AA and DHA, contents of human milk are quite variable and dependent on maternal diet (36,37).

Despite differences in the DHA and AA content of plasma lipids incident to LNA intake, a statistically significant difference in the DHA content of erythrocyte phospholipid was not observed prior to 120 d of age, and the percentage of AA in this fraction at 120 d was higher in all groups than shortly after birth. This suggests that the early differences in plasma phospholipid contents of these fatty acids among groups may have limited physiological relevance, particularly if the fatty acid pattern of erythrocyte phospholipid more closely reflects the pattern of tissue lipids than the fatty acid pattern of plasma lipid as suggested by animal studies (38,39) as well as recent postmortem studies in infants (12–14,40).

The lack of difference in the AA content of the erythrocyte phospholipid fraction reported here is in agreement with the findings of Ponder *et al.* (8) in the erythrocyte phosphatidylcholine and phosphatidylethanolamine fractions of term infants fed LA/LNA ratios of 39 vs. 7 and Clark *et al.* (7) in the erythrocyte total lipid fraction of infants fed LA/LNA ratios of 19.2 vs. 3.8. In addition, postmortem studies have shown no difference in brain AA content of breast-fed vs. formula-fed infants (12–14). Taken together, these findings suggest that supplementing infant formulas with only DHA might result in an erythrocyte (and presumably other tissue) lipid fatty acid pattern, although not a plasma lipid pattern, similar to that of breast-fed infants. However, Auestad *et al.* (41) recently reported that addition of DHA (0.23% of total fatty acids) to a formula containing 20.7% of fatty acids as LA and 1.9% as LNA (LA/LNA = ~11) resulted in a decrease in the AA content of erythrocyte phosphatidylethanolamine.

Finally, although the study reported here did not directly address the issue of whether formulas for term infants should be supplemented with DHA and/or AA, the data reported contribute to the growing body of evidence, suggesting that supplementation with one or both fatty acids will be required to achieve plasma and tissue lipid contents of both DHA and AA similar to those of breast-fed infants. Equally important, the data suggest that the amounts of AA and/or DHA needed to achieve either goal will depend upon the relative amounts of the precursors of both fatty acids in the formula. It is important to emphasize that the LA/LNA ratios of the formulas evaluated in this study were achieved by altering only LNA content. Achieving the same intake ratios by altering only LA intake, theoretically, should produce the same results; however, this remains to be proven.

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Is Dietary Docosahexaenoic Acid Essential for Term Infants?[1]

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ABSTRACT: There is a need to determine whether there is a dietary requirement for docosahexaenoic acid (DHA, 22:6ω3) by term infants to achieve their full developmental potential. Studies of brain fatty acid composition have demonstrated that infants who were breast fed have greater levels of cerebral cortex DHA than infants who were formula fed, suggesting that DHA in the cerebrum is dependent on a supply in the diet. Some physiological studies report that electrophysiological and behavioral assessments of visual function are improved in breast-fed infants relative to those fed formula, and that this is related to the level of DHA in their erythrocytes, whereas other studies demonstrate equivalent visual function between breast- and formula-fed infants. However, randomized studies of DHA supplementation of infant formula demonstrate that the visual function of formula-fed infants can be improved to breast-fed levels by adding DHA to formula. Further work is necessary to establish if there are long-term benefits of dietary DHA to the term infant.

Lipids 31, 115–119 (1996).

The definition of essential fatty acid (EFA) requirement in infancy is controversial. Classically, a nutrient requirement or recommended daily allowance (RDA) is defined as the daily intake of a specific indispensable nutrient that would prevent 98% of a healthy population from developing a deficiency syndrome that could only be reversed by the addition of that nutrient to the diet. Such dietary deficiency syndromes are severe and usually life-threatening. For example, the RDA for vitamin C is set to prevent the development of scurvy, and the RDA for thiamine (vitamin B_1) is set to protect against beriberi. For today's Western society, the relevance of such conservative estimates of dietary adequacy may be questionable as issues relating to intake of specific nutrients are more likely to revolve around the question of the intake necessary to fulfill full genetic potential. Such challenges are particularly pertinent to the field of fatty acid nutrition, as much controversy has surrounded the definition of EFA requirements in infancy.

Linoleic acid (LA; 18:2ω6) used to be considered the only EFA that must be supplied in infant diets to prevent functional abnormalities. The symptoms of classic EFA (LA) deficiency include scaly, dry skin lesions, failure to thrive, and growth retardation. Such cases of EFA (LA) deficiency have only been reported in patients maintained on lipid-free intravenous nutrition (1,2) or in infants consuming skim milk-based diets (3). In all cases, the deficiency symptoms were reversed by the addition of LA to the diet. Cuthbertson (4) argued that the minimum requirements for EFA (LA) were less than 1% of total energy. If one strictly defines a dietary requirement as a minimum level of intake to avoid the development of a deficiency syndrome (as in the RDA model), then Cuthbertson's (4) assertion is probably true.

In contrast to LA, α-linolenic acid (ALA; 18:3ω3) deficiency in humans has not been extensively documented, and most clues have come from the animal literature. For example, ALA deficiency produces alopecia and scaly dermatitis in Capuchin monkeys (5) and loss of visual acuity in young Rhesus monkeys (6). In humans, ALA deficiency has been described only in one six-year-old girl presenting with neurologic and visual symptoms (7), and in five adult patients presenting with cutaneous symptoms following either prolonged intravenous nutrition (8) or long-term gastric tube feeding (9). However, in all these cases, the ALA symptoms may be partly accounted for by other nutritional deficiencies: for example, low levels of vitamin E or total EFA deficiency. The authors estimate a daily ALA requirement corresponding to 0.3–0.5% of energy to avoid deficiency symptoms (7–9).

However, over the last decade, much research interest had focused on the longer chain products of LA and ALA metabolism, as it is these metabolites that are believed to be of physiological importance. For example, arachidonic acid (AA; 20:4ω6) and eicosapentaenoic acid (EPA; 20:5ω3) are needed as indispensable precursors for the synthesis of eicosanoids (prostaglandins, thromboxanes, and leukotrienes) that operate as regulators of various cell and tissue functions. Furthermore, during perinatal development, large amounts of long-chain polyunsaturated fatty acids (LCPUFA), such as docosahexaenoic acid (DHA; 22:6ω3) and AA, are incorporated into membrane phospholipids of all tissues (10–13). LCPUFA composition of membranes is of structural and functional importance as it contributes to the modulation of

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; EFA, essential fatty acid; LA, linoleic acid; LCPUFA, long-chain polyunsaturated fatty acids; RDA, recommended daily allowance; VEP, visual evoked potential(s).

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membrane properties such as fluidity, permeability, and activity of membrane-bound receptors and enzymes (14). The highest LCPUFA concentrations are found in synaptosomal membranes and photoreceptors, and most of these LCPUFA are deposited in the last trimester of pregnancy and during early life (12,13). A number of animal studies have demonstrated a close association between perinatal EFA supply and ω3 LCPUFA contents of neural lipids, and to the development of visual function (6,15). A similar relation between ω3 LCPUFA and visual function also has been reported for preterm infants, resulting in expert nutrition committees recommending that preterm formulas be supplemented with LCPUFA (16,17), as LCPUFA, such as DHA and AA, occur in breast milk but are not contained in most formulas. Such recommendations are based on optimizing performance of preterm infants and, hence, defining a "sufficiency state" rather than preventing deficiency as unsupplemented infants manage to survive without major complications.

However, the definition of a sufficient state for the term infant is highly debatable. Recent studies comparing the fatty acid status and visual function of breast- and formula-fed term infants have reported mixed results (18–21), and there is an apparent reluctance to accept that the term infant may be as vulnerable as the preterm infant, especially as term infants grow and thrive on current formulas. The scope of this paper is to review and update the biochemical and physiological evidence that may implicate a role for DHA in the optimal development of the healthy, term infant.

Brain composition. It is perhaps not suprising that dietary fat composition has an effect on the fatty acids that are accumulated in brain tissue of human infants during the first year of life as the weight of the brain of infants is reported to increase by 750 g (22). Although much of this weight gain is thought to be due to myelination (thus chiefly involving the accumulation of saturated and monounsaturated fatty acids), a large degree of nerve cell proliferation also occurs involving cell membranes rich in LCPUFA. However, the results of early animal studies, particularly those with weanling rats, lead to a belief by some that young brains were impervious to change by dietary fats (23). More recent results (24) have highlighted the possibility that compositional change of brain tissue is only possible during a distinct period (or window) soon after birth.

We have previously reported that the lipids of the cerebral cortex taken from breast-fed infants were higher in DHA than in infants fed formula. These results have been confirmed by other investigators (22,25). We have continued to analyze samples of tissue as they became available (Fig. 1). Our current results confirm that accumulation of DHA in the cerebral cortex of breast-fed infants continues for at least 40 wk of life, increasing from a value of about 7.5 to about 10% of total fatty acids (Fig. 1A). Although our earlier results (26) indicated no change in birth levels of formula-fed infants, the increased sample size makes it clear that DHA also accumulates in formula-fed infants, but at a much reduced rate (from about 7 to about 8% total fatty acids in 40 wk) relative to breast-fed infants.

Levels of AA also change dramatically after birth, increasing in a curvilinear manner from approximately 9 to 12% of total fatty acids in the first 20 wk of life (Fig. 1B). Interestingly, these changes are independent of diet, indicating that either AA is aggressively accumulated, or that synthesis of AA from its 18 carbon precursor, LA, is sufficient to meet the requirement of the growing infant brain. Another way of phrasing this is that the dietary AA provided in breast milk may be redundant as far as the biochemistry of the brain is concerned, and that the lower AA levels in the blood of formula-fed infants may have limited relevance.

The percentage of LCPUFA does not increase in all areas of the brain in the first year of life. In the brain stem there is a continuous depletion in the proportions of both DHA (Fig. 1C) and AA (Fig. 1D) which is independent of diet. These results are consistent with the myelination that is known to occur in the brain stem during this period, and reflected by the increasing levels of monounsaturated fatty acids ($r^2 = 0.53$, $P < 0.001$).

It is important to stress that the infants from which brain samples were taken in both our studies and that were reported by Farquharson and co-workers (22,25) were all term infants. In particular, the infants in our studies all died suddenly and were apparently well immediately prior to death. Thus, the spectrum of fatty acids observed in these subjects is likely to reflect the pattern of the brains of living infants of equivalent ages. Even though term infants have presumably benefited from a full placental supply of LCPUFA, brain growth continues postnatally and the brain remains plastic to compositional change for at least 40 wk.

The fundamental importance of these results is that the level of DHA of the cerebrum is dependent on a supply of DHA in the diet. Providing only ALA in the diet, at least at the levels provided in current infant formulas, appears to be inadequate to support maximum deposition of DHA in the brain of the developing term infant. It is reasonable to postulate that these diet-induced changes in the brain cortex composition may have some physiological sequelae.

Breast vs. formula-fed infants. Our initial work suggested that there was a measurable functional difference between breast- and formula-fed infants (18). In this study (18), visual evoked potential (VEP) acuity was examined as a measure of neural maturity of the visual pathway of healthy term infants fed either breast milk or formula, along with their polyunsaturated fatty acid status. We observed that breast-fed infants had better VEP acuity than formula-fed infants, and that there was also a correlation ($r^2 = 0.42$, $P < 0.01$) between VEP acuity and erythrocyte DHA (marker of DHA tissue status). Thus, formula-fed infants had low DHA levels and reduced visual acuity, whereas breast-fed infants had high DHA levels and increased acuity.

Some other studies comparing breast and formula feeding have demonstrated comparable results (Table 1). Birch and co-workers (19) showed superior visual acuity as assessed by both VEP and forced choice preferential looking in breast-fed compared with formula-fed infants at four months of age. The

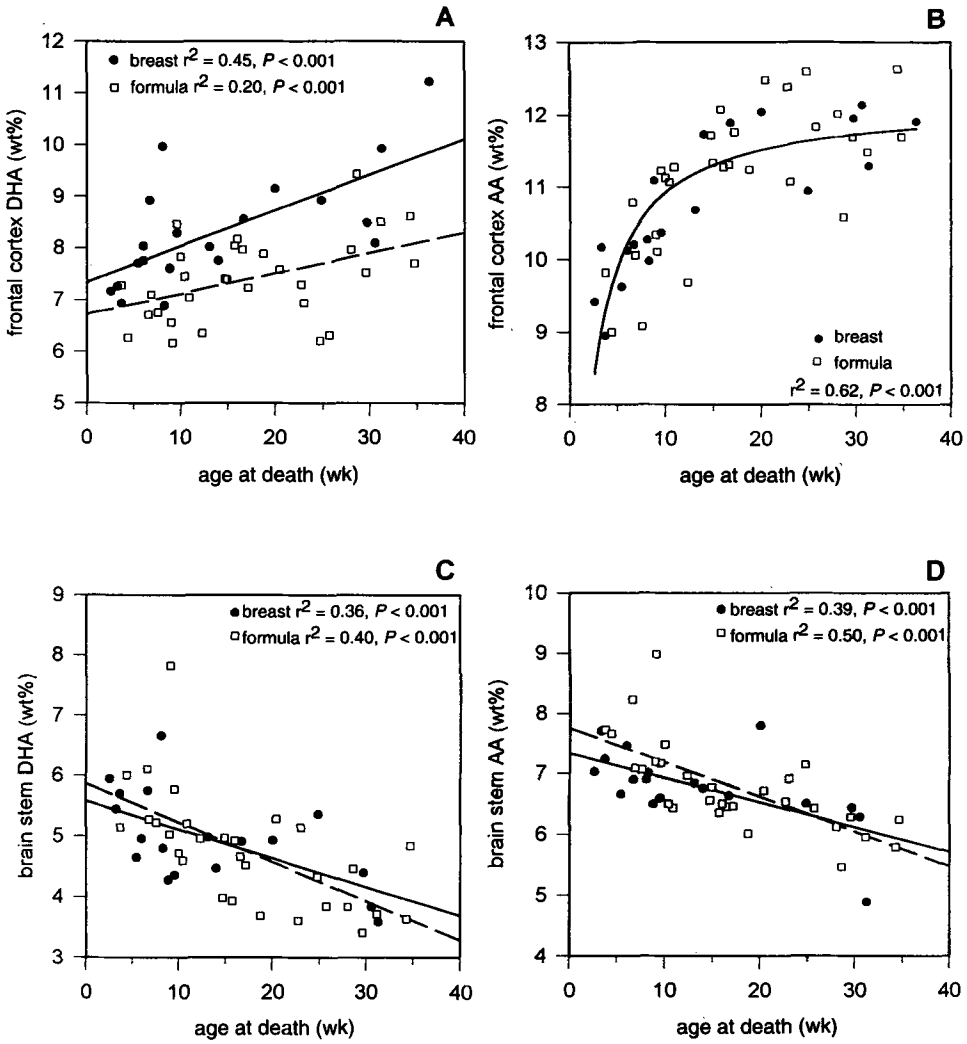


FIG. 1. Levels of docosahexaenoic acid (DHA; A,C) and arachidonic acid (AA; B,D) in brain frontal cortex (A,B) and brain stem (C,D) taken from term infants who died suddenly plotted as a function of age at death. Breast-fed infants (n = 21 frontal cortex; n = 19 brain stem) are defined as those receiving breast milk for ≥85% of their lives. Formula-fed infants (n = 30) are defined as those receiving breast milk for <30% of their lives.

TABLE 1
Effects of Breast- and Formula-Feeding on Visual Development of Term Infants[a]

Authors	Treatments	Parameters assessed	Timing of assessments	Effects
Makrides *et al.* (Ref. 18)	HM (n = 8) formula (n = 8) LA/ALA = 12–15:1–1.6	Transient VEP acuity	22 wk	logMAR: HM < formula fed
Birch *et al.* (Ref. 19)	HM formula (corn oil base) (n = 30 total)	Steady-state VEP acuity	4 mon	logMAR: HM < formula fed
		Forced choice preferential looking acuity	4 mon	logMAR: HM < formula fed
Jorgensen *et al.* (Ref. 20)	HM (n = 9) formula (n = 18) LA/ALA = 8:1 from 1 mon	Preferential looking acuity	1, 2, and 4 mon	Log (cycles/degree[b]): HM > formula at 2 and 4 mon
Innis *et al.* (Ref. 21)	HM (n = 17) formula (n = 18) LA/ALA = 17.9:2.1	Preferential looking acuity by rapid card method	14 d and 3 mon	HM = formula fed

[a]LA, linoleic acid; ALA, α-linolenic acid; HM, human milk; VEP, visual evoked potential; MAR, minimum angle of resolution. [b]Note that higher cycles/degree implies better acuity and a smaller MAR implies better acuity.

mean VEP acuity of the breast-fed group was 0.51 log units compared with 0.62 log units for the formula-fed group ($P < 0.05$). Similarly, forced choice preferential looking acuity was also better in breast-fed rather than in formula-fed infants (0.73 vs. 0.81 log units, $P < 0.05$). These results were confirmed in a study by Jorgensen *et al.* (20) that demonstrated improved behavioral acuity at two and four months in exclusively breast-fed infants over those that were predominantly fed infant formula, despite the fact that the latter group were initially breast fed for some weeks (<4 wk). In contrast, Innis and co-workers (21) failed to detect any differences in visual acuity between breast- and formula-fed term infants at three months of age. It may be that the differences in these results will eventually be explained by variations in methodologies chosen to assess visual function. Relative to behavioral methods, VEP acuity has lower (better) acuity thresholds and tends to mature more rapidly (27,28). Alternatively, the disparity in the results may be explained by differences in the availability of ALA (the DHA precursor) in the various formula fat blends tested; or other confounding influences, such as gender, parental education, and socioeconomic status, none of which are accounted for in these studies.

However, the association between acuity or development and mode of feeding provides no evidence of causation. Randomized studies comparing the visual and neural development of infants fed formula supplemented with and without DHA are necessary for a causative link between dietary DHA and visual development in healthy, term infants to be established. Indeed, data from such studies are emerging from a number of research centers and are providing unique information regarding the potential role of DHA in a "complete and sufficient" diet for infants born at term.

Randomized studies of ω3 LCPUFA supplementation. Two randomized trials of ω3 LCPUFA supplementation of infant formula have been reported in term infants (see Table 2). In both studies, mothers who chose to formula feed their infants from birth were randomized to receive either a supplemented or placebo formula, and were compared with a reference group of breast-fed infants. In our study (29), VEP were performed under transient conditions at 16 and 30 wk, and VEP acuity was determined. Breast-fed infants and those supplemented with 0.36% DHA (in the form of a fish and evening primrose oil blend) had significantly better acuities than infants fed standard formula at both 16 ($P < 0.001$) and 30 ($P < 0.01$) weeks of age. These results provided no evidence of "catch-up" with age and are consistent with the visual loss observed in the rhesus monkey model of ω3 fatty acid deficiency (6). Carlson *et al.* (30) also demonstrated improved acuity, as assessed by the Teller method, in infants fed breast milk or DHA-supplemented formula compared with infants fed standard formula at two months of age. Unlike the results of our study, the effect of DHA supplementation was not evident in older infants. This may be explained by the different nature of the chosen assessments, or alternatively, by the differences in availability of ω3 fatty acids in the two test formulas.

In summary, both studies provide evidence for a functional role for dietary DHA in healthy, term infants. Interestingly, both studies measured effects on different aspects of visual function, with the VEP providing a visual sensory threshold measured at the occipital cortex and the behavioral (Teller) acuity requiring the integration of neural processes beyond the occipital (visual) cortex. Indeed, biochemical data on cerebral cortex fatty acid composition is consistent with the assertion that DHA is important for brain function. We sampled cerebral cortex tissue from the anterior frontal lobe (26), and Farquharson *et al.* sampled parietal lobe (22,25). Both these brain areas are associated with integrative neural processes and functions, suggesting that the dietary effects of DHA may not be limited to the visual pathway. Further study of fatty acid composition of specific brain loci may provide clues as to the types of physiological functions that may be affected. Such studies are necessary to complement and validate the results of randomized clinical trials in infants.

Since the preparation of this manuscript, two other randomized studies of LCPUFA supplementation of infant formula for term infants have been published (31,32). Agostoni

TABLE 2
Randomized Studies on the Effects of ω3 Fatty Acids on Visual Function of Term Infants[a]

Authors	n	Treatments	Parameters assessed	Timing of assessments	Effects
Makrides *et al.* (Ref. 29)	23 19 13	HM A - LA/ALA = 16.8:1.6 B - LA/ALA = 17.4:1.5 + 0.36% DHA + 0.58% EPA + 0.27% GLA	Transient VEP acuity	16 and 30 wk	logMAR: HM, B < A
Carlson *et al.* (Ref. 30)	19 20 19	HM (for at least 3 mon) A - LA/ALA = 22:2.2 B - LA/ALA = 22:2.0 + 0.12% DHA + 0.40% AA	Preferential looking acuity by rapid card method	2, 4, 6, 9, and 12 mon	acuity (cycles/degree[b]): HM, B > A at 2 mon HM = A = B at 4, 6, 9, and 12 mon

[a]DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; AA, arachidonic acid; See Table 1 for other abbreviations.
[b]Note that higher cycles/degree implies better acuity and a smaller MAR implies better acuity.

et al. (31) report that term infants fed a formula supplemented with DHA and AA from an egg phospholipid source have a higher developmental quotient than unsupplemented infants. Conversely, Auestad and co-workers (32,33) demonstrated no effect of LCPUFA supplementation on visual, mental, or psychomotor development of term infants.

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REVIEW

Trans Unsaturated Fatty Acids in Bacteria

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ABSTRACT: The occurrence of *trans* unsaturated fatty acids as by-products of fatty acid transformations carried out by the obligate anaerobic ruminal microflora has been well known for a long time. In recent years, fatty acids with *trans* configurations also have been detected in the membrane lipids of various aerobic bacteria. Besides several psychrophilic organisms, bacteria-degrading pollutants, such as *Pseudomonas putida*, are able to synthesize these compounds *de novo*. In contrast to the *trans* fatty acids formed by rumen bacteria, the membrane constituents of aerobic bacteria are synthesized by a direct isomerization of the complementary *cis* configuration of the double bond without a shift of the position. This system of isomerization is located in the cytoplasmic membrane. The conversion of *cis* unsaturated fatty acids to *trans* changes the membrane fluidity in response to environmental stimuli, particularly where growth is inhibited due to the presence of high concentrations of toxic substances. Under these conditions, lipid synthesis also stops so that the cells are not able to modify their membrane fluidity by any other mechanism.
Lipids 31, 129–137 (1996).

OCCURRENCE OF *TRANS* FATTY ACIDS IN PROKARYOTES

In contrast to eukaryotic cells, bacteria usually do not have reserve fats in the form of triacylglycerols, and their fatty acids are only found as components of membrane lipids. If grown under standardized conditions, bacteria possess a constant fatty acid composition which is specific for a genus or even a species. Because membrane fatty acids can be extracted and measured with relatively simple methods (1,2), the identification of bacteria with the help of their fatty acid composition as a biochemical marker has become a routine method (3–7).

Whereas Archaebacteria mainly have isoprenoid compounds instead of fatty acids as constituents of their membrane lipids (8), the well-known spectrum of bacterial fatty acids comprises saturated, *cis*-monounsaturated, cyclopropane, and methyl-branched fatty acids. During the last years, two new fatty acid types have been found as membrane constituents of prokaryotes—polyunsaturated fatty acids (9–11) and a completely novel class of fatty acids, *trans* configurated unsaturated fatty acids. Even some years ago, the *trans* configuration of the double bond was described as an exception usually not found in unsaturated fatty acids of bacteria (12). One reason for this development may have been the introduction of capillary columns into gas-chromatographic techniques which led to a much better differentiation between fatty acid methyl esters (13,14).

It has long been known that the rumen and the feces of animals and humans contain high amounts of *trans* fatty acids. Apparently, hydrogenation of multiple unsaturated fatty acids from plant material by the ruminal and intestinal bacterial flora is responsible for this occurrence (15). It is well documented that bacteria like *Eubacterium lentum* reduce different unsaturated fatty acids, such as linolenic and linoleic acid, to yield largely stearic acid and some *trans* acyl derivatives (16,17). The physiological reasons for these fatty acid transformations in the anaerobic environment are still unknown. It has been reported that the polyunsaturated fatty acids are more toxic to the anaerobic bacteria than are the products of hydrogenation. Therefore, one function of bacterial fatty acid transformations may be to destroy growth-inhibiting substances (17). Another suggestion is that this process enables the strictly anaerobic bacteria to dispose excess reducing power (15). This bacterial activity is also responsible for the occurrence of *trans* fatty acids in cow's milk and milk products such as butter. Depending on the diet, milk fat has 2–9% total *trans* fatty acid isomers. Besides the consumption of artificially-derived fats as hydrogenated vegetable oils, the uptake of these bacterial transformation products leads to the presence of *trans* fatty acids in humans. The risk of dietary intake of *trans* isomers for human health, including coronary heart disease, is a matter of considerable controversy (18–24).

In several respects, the ruminal transformation process is different from the synthesis of *trans* monounsaturated fatty acids as membrane constituents by aerobic bacteria presented in the later part of this review. Differences in the enzymatic mechanism are also apparent, as the anaerobes always shift the position of the double bond during fatty acid conversion (25). Unesterified polyunsaturated fatty acids are the substrates of the hydrogenation, and the products of this process are fatty acids with free carboxyl groups. In minor amounts, these *trans* fatty acids are also found esterified in the membrane lipids of ruminal microorganisms. However, this is ap-

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TABLE 1
Occurrence of *trans* Unsaturated Fatty Acids in Bacterial Lipids

Organism	*Trans* content[a]	18:1 *Trans* isomers Δ11	18:1 *Trans* isomers Δ9	Biosynthesis route	References
Pseudomonas atlantica	22–35	+	–	Anaerob.[b]	27
P. putida	10–41	+	–	Anaerob.	28–32
P. aeruginosa	1–41	–	+	Aerob.[c]	33
P. aureofaciens	ND[d]	+	–	Anaerob.	34
Alcaligenes sp. A7-2	1–9	+	–	Anaerob.	35
Desulfomonile tiedjei	0.3–1.1	–	–	Anaerob.	6
Anabaena sp.[e]	5	–	+[f]	Aerob.	5
Nostoc sp.[e]	3.4	–	+	Aerob.	5
Shewanella putrefaciens	1–10	+[f]	+[f]	Aerob.+ anaerob.	11
Methylococcus luteus	17.8	–	–	Anaerob.	4
Methylosinus trichosporium	6.2–9.6	+	–	Anaerob.	4,13,14
Methylomonas agile	28.2	–	+	ND	3
Vibrio cholerae	1–23	+	+[f]	Aerob. + anaerob.	36,37
Vibrio sp.	4–16	–	–	Aerob.	9,10
Vibrio sp. ABE-1	1–19	–	–	Aerob. + anaerob.	38–41
Aerococcus viridans	ND	–	–	ND	42
Arcobacter cryaerophila	ND	–	–	ND	42
Flavobacterium breve	ND	–	–	ND	42

[a]% of total fatty acid content. [b]Anaerobic way of fatty acid synthesis. [c]Aerobic way of fatty acid synthesis. [d]No data available. [e]Mean value for ten examined strains. [f]In traces (i.e., <1%).

parently not caused by bacterial biosynthesis of fatty acids as phospholipid constituents. Ruminal bacteria often are auxotrophic and have growth requirements which are partially satisfied by fatty acids or lipids. The cells well-adapted to the steady supply of these compounds take up fatty acids, including *trans* unsaturated ones, from the ruminal fluid and incorporate them into their membrane lipids.

The first description of the occurrence of *trans* monounsaturated fatty acids in aerobic bacteria was in 1978 for methane-utilizing bacteria (13) and in 1981 for a marine bacterium (26). By now, *trans* fatty acids have also been described in some other groups of bacteria (Table 1). This overview shows that *trans* unsaturated fatty acids are dispersed in different and not necessarily related groups of the prokaryotes. They are, however, predominately found in Gram-negative bacteria.

The chloroplast membranes of higher plants and algae also contain some fatty acids with a double bond of *trans* configuration (43,44). *Trans* fatty acids can be detected in minor amounts in some marine animals; these occurrences may be caused by the input and incorporation of fatty acids of microbial or plant origin into membrane lipids.

The quantity of *trans* fatty acids in bacteria can often be greater than that of *cis* unsaturated fatty acids. Similar to other fatty acids in bacteria, the relative amounts of *trans* fatty acids strongly depend on the physiological condition of the cells such as growth rate, medium composition, or environmental factors.

BIOSYNTHESIS OF *TRANS* UNSATURATED FATTY ACIDS IN BACTERIA

Basic studies on biosynthesis and function of microbial fatty acids were done in *Escherichia coli* which, normally, does not have *trans* fatty acids. However, mutants defective in unsaturated fatty acid biosynthesis incorporated *trans* fatty acids in phospholipids when supplemented as growth factors (45–47). These observations were explained by the purely structural role of these membrane constituents in establishing a lipid bilayer with controlled fluidity parameters. Ingram (48) reported that membranes of *E. coli* containing *trans* unsaturated $C_{18:1}$ fatty acids were even more tolerant to toxic ethanol concentrations than were those with the *cis* isomers. However, for the utilization of *trans* unsaturated fatty acids, in contrast to *cis* isomers, a second mutational lesion with unknown function is required in *E. coli* (49). In these studies the *trans* unsaturated fatty acids were taken up from the growth medium by the fatty acid transport systems.

Many bacterial species can accumulate fatty acids by using acyl-chain unspecific active or passive transport mechanisms. The fatty acids are oxidized *via* β-oxidation or are incorporated into membrane lipids. In *E. coli* these two processes are regulated by a common regulation system (50). Due to the possibility of uptake from the growth medium, the presence of small amounts of a special fatty acid does not indicate the existence of a biosynthesis apparatus in the cells.

In 1987, Guckert *et al.* (27) demonstrated the biosynthesis of *trans* unsaturated fatty acids *in vivo* from acetate in *Pseudomonas atlantica,* but could not explain if or how these fatty acids were formed by the known biosynthetic routes of unsaturated fatty acids. Many obligate aerobic bacteria synthesize single, unsaturated fatty acids by the so-called aerobic pathway, which is also the usual way to produce these compounds in eukaryotic cells. Here, the *cis* double bond is subsequently introduced into the aliphatic acyl chains of saturated fatty acids by specific desaturases—electrons are transported from the saturated fatty acid *via* NADPH to O_2 using a

membrane-bound short electron chain (Fig. 1A). With bacteria, these systems act on phospholipids as substrates so that the cells can change the degree of saturation of their membrane fatty acids post-biosynthetically (51–53).

The second biosynthesis route, which does not depend on O_2 and therefore is called the anaerobic pathway, is the one which is present in the majority of prokaryotic cells, namely in anaerobic, facultatively anaerobic, and also some aerobic bacteria (40,50,54). In this pathway (Fig. 1B), which has been studied in detail in *E. coli*, unsaturated fatty acids are produced in an intermediate reaction of fatty acid biosynthesis: During the elongation cycle, a *trans* double bond is produced which can further be converted in two different ways. If it is further reduced, saturated fatty acids are formed. But, a specific hydratase, β-hydroxydecanoyl-acyl carrier protein-dehydratase, can isomerize the *trans* double bond in position 2,3 of the $C_{10:1}$ fatty acid into the *cis* form now in the 3,4 position. This product cannot be reduced, and the *cis* double bond remains intact during the subsequent elongation cycles. The final saturated and *cis*-unsaturated acyl chains are then incorporated into the membrane phospholipids of the cells. There is no post-biosynthetic modification of the degree of saturation of the fatty acids.

The positions of the double bonds of microbial C_{18}-fatty acids depend on the biosynthetic route by which they are produced; whereas during the anaerobic pathway a C_2-elongation of palmitoleic acid [$C_{16:1}(c9)$] leads to *cis*-vaccenic acid [$C_{18:1}(c11)$], the aerobic pathway produces oleic acid [$C_{18:1}(c9)$]. Therefore, the occurrence of *cis*-vaccenic acid or oleic acid in bacterial membranes gives an indication of the biosynthetic route of unsaturated fatty acids which is used by the organism. Studies with *Vibrio* ABE-1 forming *trans* fatty acids indicated that here even both pathways are present and are involved in biosynthesis of unsaturated fatty acids (40). Table 1 shows that the majority of *trans* fatty acids are formed by bacteria possessing the anaerobic route of biosynthesis. Apparently, organisms featured by the aerobic pathway are also able to form these compounds. From this it can be concluded that either different pathways are involved in the biosynthesis of *trans* unsaturated fatty acids, or that the mechanism is independent from the way of synthesis of *cis* unsaturated fatty acids.

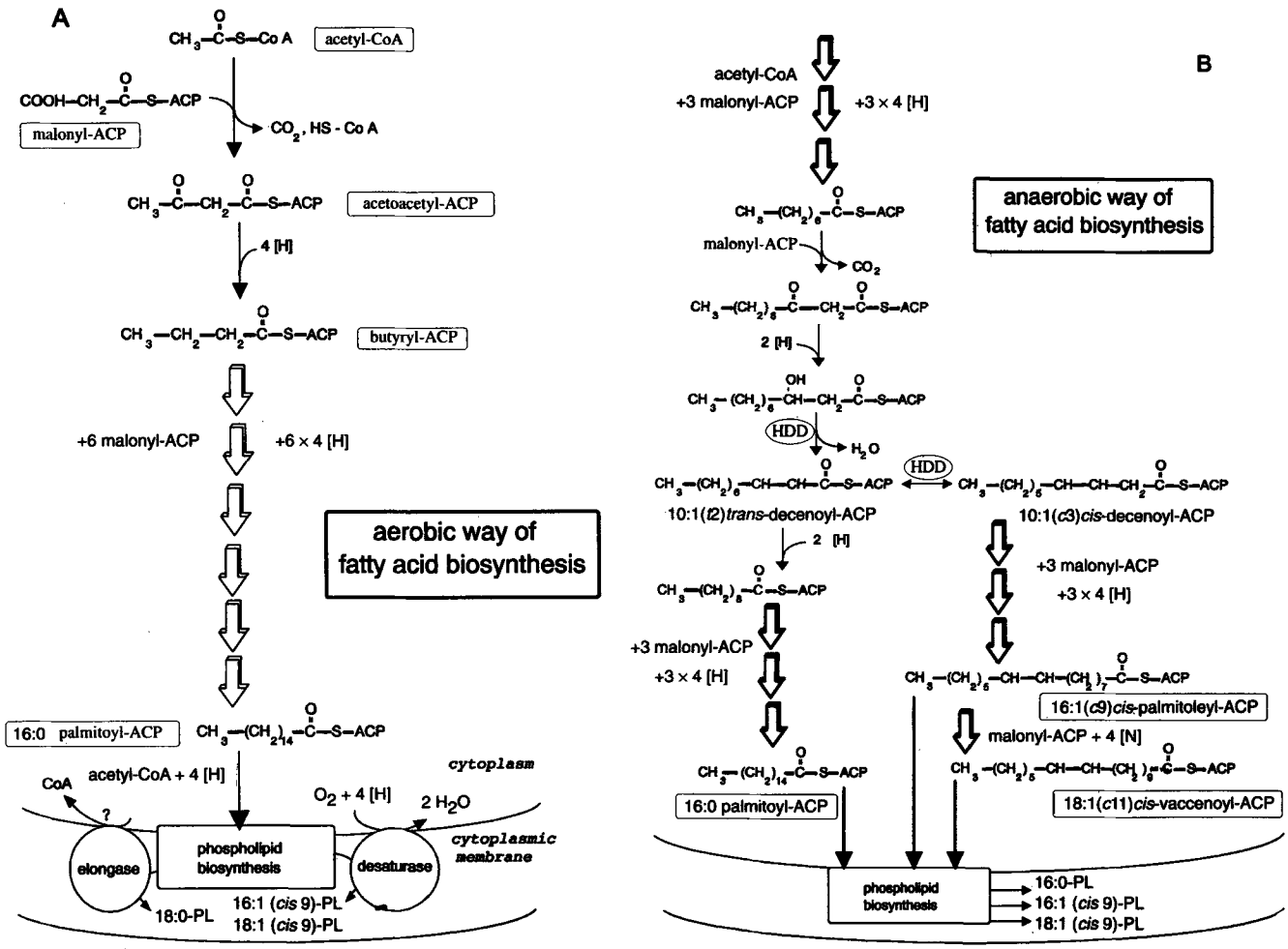


FIG. 1. Aerobic (A) and anaerobic (B) pathway of fatty acid biosynthesis and incorporation of fatty acids in phospholipids. PL, phospholipids; ACP, acyl carrier protein; HDD, β-hydroxydecanoyl-ACP-dehydratase.

Because of the position of the *trans* double bond in the intermediates of the anaerobic pathway [$C_{10:1}(t2)$], a further elongation of these acyl chains would lead to *trans* unsaturated fatty acids with double bonds in positions 8 (for $C_{16:1}$) or 10 (for $C_{18:1}$). Neither form of *trans* unsaturated fatty acids has been identified in bacteria. This indicates that *trans* fatty acids are not formed by a modification of the known anaerobic pathway. In all organisms studied up to now, the position of the *trans* double bond was identical with that of a *cis* one found in the same organisms.

TRANS UNSATURATED FATTY ACIDS ARE SYNTHESIZED BY A DIRECT ISOMERIZATION OF THE *CIS* FATTY ACIDS IN THE CELL MEMBRANE

The mechanism of synthesis of *trans* unsaturated fatty acids has been studied in detail with cells of *P. putida* and in the *Vibrio* strain ABE-1.

Growing cells of *P. putida* react to the addition of phenol or chlorinated phenols with a concentration-dependent increase in *trans* fatty acids and with a simultaneous decrease in the corresponding *cis* unsaturated fatty acids (29). The conversion also occurs in nongrowing cells where the ratio between saturated and unsaturated fatty acids and the total amount of *cis* and *trans* fatty acids cannot be changed due to the cessation of lipid biosynthesis. Moreover, the reaction takes place in cells in which fatty acid biosynthesis was totally inhibited due to the addition of cerulenin (28,30). The *cis–trans* conversion reaches its final *trans*-to-*cis* ratio about 30 min after the addition of a phenolic substance. The same rate of conversion was observed in the presence of chloramphenicol, indicating that the observed system is constitutively present and does not need a genetic induction (29,30).

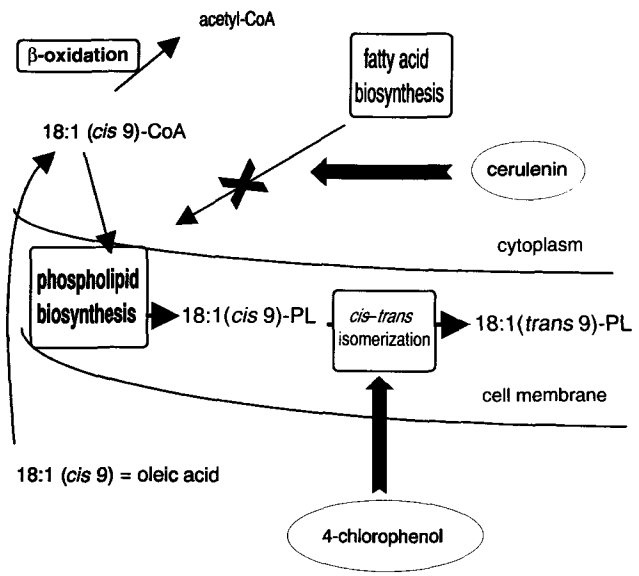


FIG. 2. *In vivo* formation of elaidic acid from oleic acid as proof of direct isomerization of *cis* to *trans* unsaturated fatty acids in *Pseudomonas putida* P8 induced by 4-chlorophenol. Abbreviation as in Figure 1.

Pseudomonas putida supplemented with oleic acid [$C_{18:1}(c9)$], which normally is not synthesized by this strain, incorporated it into the membrane lipids (Fig. 2). After adding 4-chlorophenol, which induced isomerization of fatty acids, oleic acid was converted into the *trans* isomer of this acid, elaidic acid [$C_{18:1}(t9)$]. These experiments were also done with radioactively-labelled oleic acid and in cells in which fatty acid biosynthesis was inhibited by cerulenin (55). The absence of other radioactive compounds in these experiments also indicated that oleic acid was not degraded or converted to other fatty acids. From these results, it can be concluded that *trans* fatty acids are synthesized by a direct isomerization of *cis*- to *trans*-unsaturated fatty acids without a shift in the position of the double bond. A similar observation has been made with marine psychrophilic *Vibrio* strains. Here the *cis–trans* isomerization was observed after an increase in the ambient temperature, and the position of the double bond was also not shifted (38,39,41). In experiments with isolated membrane fractions, the isomerization after addition of 4-chlorophenol was located in the inner membrane fraction, whereas those of the outer membrane were not active (55). These investigations showed that *trans* fatty acids are synthesized by a direct isomerization of the double bond of unsaturated fatty acids in the plasma membrane. Because the isomerization takes place in isolated membrane vesicles, the substrates for this reaction must be the acyl chain of the phospholipids. That means that the matrix of this still unknown system is also its substrate.

As in *P. putida,* the major phospholipids of the *Vibrio* strains were phosphatidylethanolamine and phosphatidylglycerol, both containing *trans* fatty acids. The positional distribution of these fatty acid components was also determined. They were located at both positions, but mainly at position *sn*-2 of the glycerol moiety (10,39,56).

In contrast to the system described here, all other known microbial or eukaryotic *cis–trans*-isomerases shift the position of the double bond during the isomerization reaction (25). In *E. lentum* the cytoplasmic isomerase converting *cis*- into *trans* unsaturated fatty acids during hydrogenation of fatty acids was characterized in detail. This system was inhibited by sulfhydryl reagents, e.g., *N*-ethylmaleimide (17,25). Experiments with *P. putida* S12 could show that the isomerization system in these cells is insensitive for *N*-ethylmaleimide (H.J. Heipieper, unpublished results). Obviously, the observed isomerization mechanism is a totally new enzymatic system. No biochemical or genetical characterization of the membrane system has been reported as yet.

PHYSIOLOGICAL FUNCTION OF *TRANS* UNSATURATED FATTY ACIDS IN BACTERIA

The first interpretation of the physiological function of *trans* unsaturated fatty acids was done by Guckert *et al.* (36). In long-term experiments with *Vibrio cholerae,* they showed that the amount of *trans* unsaturated fatty acids was increased in starving cells. The authors explained this by the greater sta-

bility of the *trans* fatty acids compared with the *cis* isomers. During starvation, the cells would metabolize their fatty acids, whereby the *cis*-fatty acids were better degraded than the *trans* isomers.Therefore, Guckert *et al.* (36) have suggested using a *trans/cis* ratio of greater than 0.1 in environmental samples as an index for starvation or stress. But this theory is not in agreement with results of studies with *P. putida* and *Shewanella putrefaciens*, where cells grown in batch cultures had a much lower content of *trans* unsaturated fatty acids when they were in the stationary growth phase (11,28). Therefore, the function of *trans* unsaturated fatty acids as a reserve for starvation seems to be unlikely.

Investigations concerning the toxic effects of organic compounds on cells of *P. putida* may help to elucidate the reason for *trans* unsaturated fatty acids occurring in these bacteria. The main target for the toxicity of organic solvents to bacterial and eukaryotic cells is the cell membrane (57–59). The compounds partition preferentially in membranes, causing an increase in membrane fluidity which is leading to a nonspecific permeabilization (57,58,60–64). For species of the genus *Pseudomonas,* it has often been observed that they are able to adapt to very high concentrations of inhibitory substances, leading to the ability to grow in the presence of organic solvent (65–67). The most important adaptative reaction of these bacteria against membrane active substances is changes in the fatty acid composition of membrane lipids. Figure 3 shows growth inhibition and membrane modifications of cells of *P. putida* in the presence of different concentrations of 4-chlorophenol. At concentrations up to 300 mg/L, which totally inhibits growth, the cells increase the degree of saturation of their membrane fatty acids. At higher concentrations, the cells do not use this mechanism but increase the content of *trans* unsaturated fatty acids. To explain this observation, the function and regulation of membrane fluidity in bacteria have to be described in more detail.

The physical properties of biological membranes, which can occur either in a highly ordered or a fluid crystalline phase state, are determined by the composition of the membrane lipids and fatty acids (47,68). The cellular barrier is simultaneously the matrix for enzymes, which depend on its fluidity conditions (62,63). On the other hand, some environmental factors, like temperature and organic solvents, have a strong influence on membrane fluidity and, therefore, on the physiological properties of a membrane. Microorganisms, however, can adapt to changes of their membrane fluidity; they react to externally dictated (occurring) changes in their environment by modifying their membrane to maintain a constant degree of fluidity. This mechanism is called "homeoviscosic adaptation" (52,62,69–72).

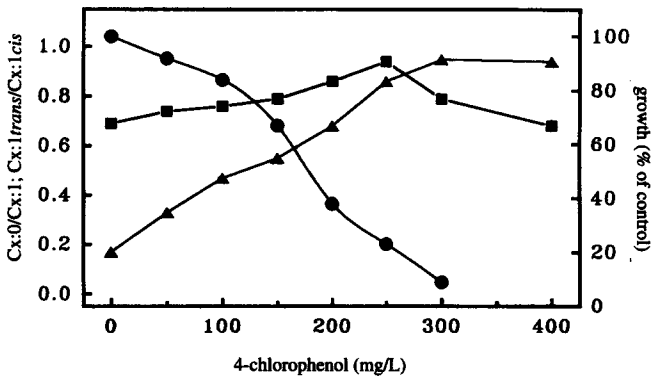


FIG. 3. Effect of 4-chlorophenol on cellular growth (●), degree of saturation (■), and *trans/cis* ratio (▲) of fatty acids of *Pseudomonas putida*.

Eukaryotic cells mainly regulate the fluidity of their membranes by changes of the cholesterol content. Bacteria, which normally do not possess steroids, have to use other mechanisms. Most bacteria regulate the fluidity of their membranes by varying the degree of saturation of their membrane fatty acids (51,69,73).

In membranes with a high content of saturated fatty acids, the acyl chains of the fatty acids produce the optimal hydrophobic interaction with each other which leads to a highly packed, rigid membrane. Due to the free rotation about the C–C bonds, saturated acyl chains can occupy an *all-trans* conformation, which allows the chain to be maximally extended. This conformation is preferred if the fluidity of a membrane is low. The temperature for the transition from the gel to the liquid-crystal membrane phase increases with an increasing ratio of saturated toward unsaturated fatty acids (degree of saturation). Contrary to this, the double bond of *cis* unsaturated fatty acids possesses a nonmovable bend with an angle of 30° in the acyl chain. Due to this bend, the highly ordered package of the acyl chains is disturbed, which leads to a lower phase transition temperature of these membranes (47). The transition temperature of a phosphatidylethanolamine bilayer decreases by about 42°C if only one saturated acyl chain per lipid molecule is replaced by a *cis* unsaturated one (39).

The *trans* configuration has a completely different effect on the fluidity of the phospholipid bilayer than the *cis* one (47,68,74). Figure 4 shows the structures of the two unsaturated fatty acids. *Trans* fatty acids can take up a long extended structure which is similar to that of saturated fatty acids. Membranes built of *trans* fatty acids can therefore form a more rigid package of the phospholipids than membranes with *cis* fatty acids. The measurement of phase transition temperatures of membranes built from identical phospholipids with *trans* instead of *cis* unsaturated fatty acids results in significantly higher transition temperatures(31,39,47). The replacement of one *cis* acyl chain by a *trans* fatty acid in phosphatidylethanolamine increases the transition temperature by 18–31°C, depending on the structure of the other acyl chain in the lipid molecule. Therefore, the conversion of *cis* unsaturated fatty acids into their *trans* configuration results in a significant reduction of the membrane fluidity which is, however, smaller as the replacement of *cis* by saturated fatty acids.

Bacteria such as *P. putida,* which possess the anaerobic pathway of fatty acid biosynthesis, can only change the de-

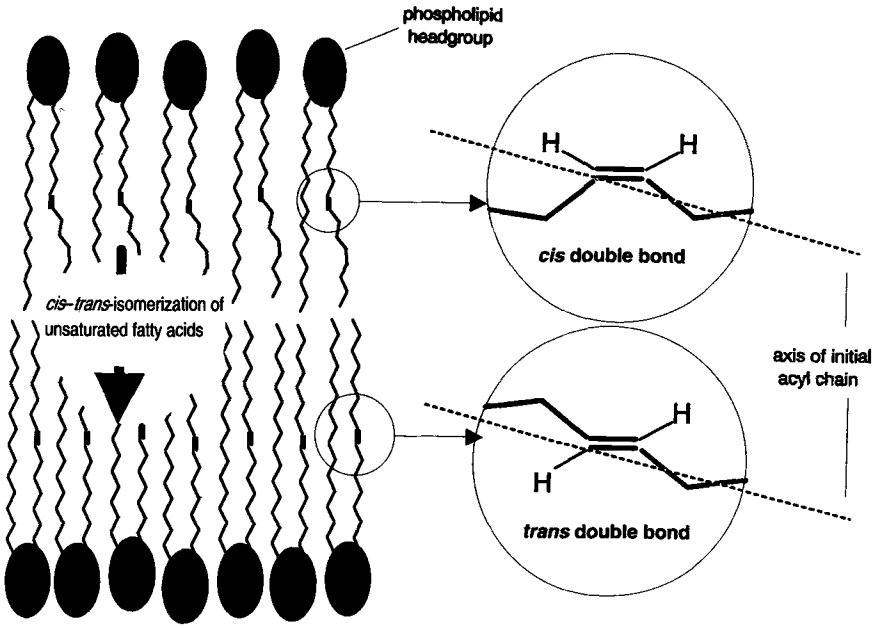


FIG. 4. *Cis-trans* isomerization of fatty acids and molecular packing of phospholipids with either *trans*- or *cis*-unsaturated fatty acids.

gree of saturation of fatty acids at the level of *de novo* synthesis. Bacterial lipids are stable cellular components, and the exchange of acyl groups of membrane lipids is very small. Fatty acid and lipid synthesis strongly correlate with cellular growth. In nongrowing cells, phospholipid synthesis is prevented by the "stringent control" and fatty acid synthesis is completely inhibited in the absence of lipid synthesis (54,75,76). Therefore, the cells are unable to change the degree of saturation of the membrane lipids, e.g., at concentrations of an inhibitor which totally prevents cellular growth (Fig. 3). Bacteria, however, which possess a system to isomerize the double bond of their unsaturated fatty acids, are still able to change the fluidity of their membranes under these adverse conditions.

The *cis–trans* isomerization is obviously an alternative mechanism of some bacteria to modify membrane fluidity. It would be of crucial importance when the regular mechanism of membrane adaption, the change in the degree of saturation, cannot be carried out due to the absence of lipid and fatty acid biosynthesis. Even when no energy substrate is available for the cells, a *cis–trans* isomerization of unsaturated fatty acids can be carried out without the high costs of energy and precursors. Another advantage of the mechanism is the speed of this direct fatty acid modification. Cells can react much faster to changes in their environment and can survive long enough so that they are able to subsequently react with long-term adaptative mechanisms.

POSSIBLE ECOLOGICAL ROLE OF *CIS–TRANS* ISOMERIZATION IN BACTERIA

This system modifying fatty acyl groups is obviously profitable to bacteria in which growth and energy-dependent adaptation mechanisms are insufficient when the physicochemical factors of their environment change. The ecological advantage for the marine psychrophilic *Vibrio* strains could be to react to changes in temperature with a system that is faster than one changing the degree of membrane saturation, because this reaction is related to the growth rates of the cells which are very slow at very low temperatures (38–40).

The other group of bacteria where the *cis–trans* isomerase was found are *Pseudomonas* strains, which play an important role as degraders of environmental pollutants. This system was found in *P. putida* strains which had the ability to degrade phenols (29), styrene (31), and toluene (31), and had the capacity to adapt to a wide range of organic compounds (66,77). Using the *cis–trans* isomerization system, the bacteria could adapt very quickly to toxic concentration of organic substrates. The cells can stabilize their membranes, allowing them to stay in physiologically acceptable condition in which they can activate (induce) other long-term adaptative mechanisms (58,73,78).

In *P. aeruginosa,* a content of *trans* unsaturated fatty acids of more than 40% could be found when these cells were grown with olive oil as carbon source (79). Olive oil possesses a high content of *cis* unsaturated fatty acids. Here the *cis/trans* isomerase seems to allow the cells to grow on this substrate, also incorporating the fatty acids from the olive oil in their membranes. Without the isomerization into the *trans* configuration, the high content of *cis* unsaturated fatty acids would markedly increase the fluidity of their membrane and the alternative, *de novo* synthesis of saturated fatty acids, is a very energy-dependent process.

A study on the effect of desiccation was carried out with a strain of *P. aureofaciens.* The cells significantly increased their ratios of *trans* to the corresponding *cis* fatty acids, whereas

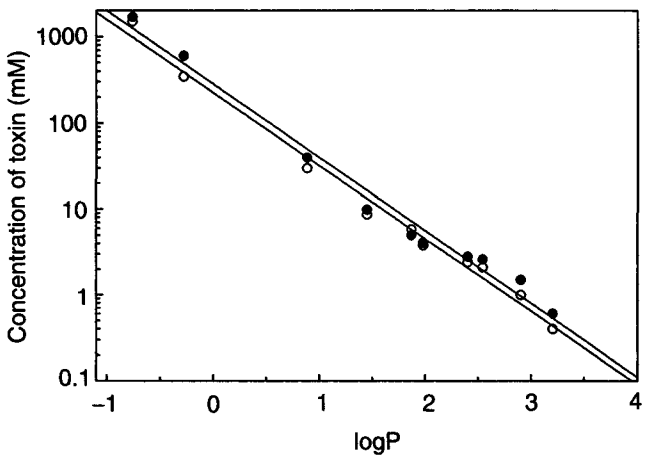


FIG. 5. Correlation between the hydrophobicity, given as the logP value of ten different organic compounds, growth inhibition, and *trans/cis* ratio of *Pseudomonas putida* cells. Growth inhibition (○) is presented as the LD 50 concentration. TC 50 (●) is the concentration of a specific organic compound at which the *trans/cis* ratio of unsaturated fatty acids is 50% of the maximal *trans/cis* value got by this inhibitor. The applied organic compounds are (in order of increasing logP values): methanol, ethanol, 1-butanol, phenol, 1-hexanol, p-cresol, 4-chlorophenol, toluene, 1-octanol, 2,4-dichlorophenol.

only a minor increase in the degree of saturation was observed as response on dry conditions (34). This may be another example where *cis–trans* isomerization substitutes the conventional mechanism of regulation of membrane fluidity if environmental conditions are adverse for the microorganisms.

Still unknown is the function of *trans* unsaturated fatty acids in methanotrophic bacteria, where they seem to be a common feature in all genera (3,4,13,14). The ecological function could be to react against high concentrations of methanol or formaldehyde, which are possible growth substrates or toxic intermediates in the oxidation of methane, and/or to adapt to other detrimental environmental influences.

PROSPECTS

In an extension of theoretical interest, the detailed assessment of the *cis/trans* fatty acid isomerase system will also have some possible applications. Potentially interesting reactions may be stereospecific transformations of unsaturated acyl compounds with the aim of synthesis of optically active hydroxy compounds. The conversion of *trans* configurated double bonds in dietary products for reasons of health or food technology is another promising application. Future research should clear up which substrates are accepted by the *cis/trans* isomerase. This may be an important question to be solved for the technical employment of the system.

In view of the characteristics of *cis/trans* isomerization affected by toxic substances, this system must be investigated for its ability to function as a toxicity test for organic environmental contaminations. Heipieper *et al.* (77) showed that a strong correlation exists between the hydrophobicity of organic solvents, their toxic potential to cells of *P. putida,* and their concentration activating the isomerization of *cis* to *trans* fatty acids (Fig. 5). From these results it may be anticipated that the isomerization of *cis* to *trans* unsaturated fatty acids in *P. putida* could function as an indicator for the toxicity of organic compounds. The development of such a test especially may have advantages in situations where growth-dependent tests cannot be performed, e.g., in natural habitats. The main field for an application of this indicator seems to be *in situ* bioremediation processes, where fatty acid profiles have importance as a marker for ecological investigations of the soil microflora (34,36,80–82). Therefore, the isomerization of *cis* to *trans* unsaturated fatty acids can be a relevant parameter for the physiological status of the microbial flora in the presence of toxic pollutants.

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ARTICLE

Increased Hepatic Δ6-Desaturase Activity with Growth Hormone Expression in the MG101 Transgenic Mouse[1]

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ABSTRACT: Growth hormone (GH) has many metabolic effects, but its mechanism(s) of action are not fully understood. We studied the short-term effects of endogenously produced GH on liver Δ6-desaturase activity and adipose and liver lipid fraction fatty acid composition in transgenic mice. MG101 transgenic mice ages 73–114 d received zinc to activate the ovine GH transgene for 7 d. Nontransgenic littermates, used as controls, also received zinc. Liver lipids were fractionated into phospholipids (PL), cholesteryl esters, and triglycerides (TG), and retroperitoneal adipose fractionated into PL and TG for fatty acid analysis. Liver microsomes were assayed for Δ6-desaturase activity. Animals expressing the ovine growth hormone transgene had a 2.5-fold higher liver Δ6-desaturase activity than controls. Arachidonate and docosahexaenoate were significantly higher in liver PL of GH transgenic animals compared to controls, but both were decreased in adipose PL in the GH animals. We conclude that increased production of GH affects both production and organ distribution of highly unsaturated fatty acids. The changes in arachidonate in various lipid pools following transgene expression may mediate the systemic actions of GH. *Lipids 31*, 139–143 (1996).

Transgenic animals carrying growth hormone (GH) genes can have increased lean body mass and growth, which is of potential benefit to meat production. However, most of these transgenic animal lines suffer from various side effects including arthritis and impaired immune and reproductive functions (1). The MG101 mouse carries the ovine GH (oGH) structural gene fused with the sheep metallothionein 1a (oMT1a) promoter (2). With this combination, the MG101 mice have a minimal level of basal expression of oGH, but high expression induced with 25 mM zinc in their drinking water (2). This strain of mice does not show apparent pathology after prolonged induction of GH. Nevertheless, these mice show marked enlargement of the liver and spleen (3) in addition to increased overall body size.

Recent studies suggest that GH stimulates hematopoiesis and modulates immune functions, although the mechanisms of these effects are unknown (4). Maddaiah and Clejan (5) reported that GH administration to hypophysectomized rats caused a rapid increase in the ratio of arachidonic acid (20:4n-6) to linoleic acid (18:2n-6) in phospholipids (PL) from liver microsomes and mitochondria. In a preceding study, 20:4n-6 in serum and liver PL was markedly increased in zinc-fed MG101 mice compared with control mice fed the same diet regimen (6). This alteration of fatty acid composition suggests an increased activity of hepatic Δ6-desaturase/elongase/Δ5-desaturase pathway utilized for 20:4n-6 synthesis. As arachidonic acid plays a key role in immune response by serving as a precursor of eicosanoids, the elevation of tissue 20:4n-6 could be in part responsible for the disorders of immune function seen in some GH transgenic animals.

Adipose tissue is another target tissue of GH, although the mechanism of action of GH on the adipocyte has not been fully elucidated. Transgenic animals expressing the GH gene generally become not only larger but leaner. This response is well documented in the MG101 transgenic mouse, which shows lower body fat when compared with controls with the same body weight (3,7). The effect of GH on adipocyte membrane fatty acid composition has not been investigated.

This study was undertaken to: (i) analyze activity of hepatic Δ6-desaturase, the rate-limiting enzyme of 20:4n-6 synthesis, and (ii) characterize the effects of GH expression in the MG101 mouse on fatty acid composition of lipid subfractions in liver and adipose.

MATERIALS AND METHODS

Animals and diets. Wild type F1 females (C57BL/6 × CBA) were mated with MG101 males hemizygous for the oMT1a-oGH fusion gene. At day 10, pups were individually identified by toe-notching, and characterized for presence of the transgene by polymerase chain reaction amplification of a unique transgene sequence from toe-notch lysate (8). The

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Abbreviations: CE, cholesteryl esters; GH, growth hormone; oMT1a, ovine metallothionein 1a; oGH, ovine growth hormone; PL, phospholipids; TG, triglycerides.

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oMT1a-oGH transgene is inherited in strict Mendelian fashion, and hemizygous progeny supplemented with zinc express the transgene due to dominant gene action. Therefore hemizygous transgenics and homozygous wild-type littermates (controls) were used as the two experimental groups. Animals were housed under conditions of 21°C, 55% humidity, and 14–10-h light–dark cycles. After weaning at day 21, the mice of both groups were fed Purina Rodent Chow 5001 (Purina Mills, St. Louis, MO) and water *ad libitum*. Groups of mature female mice (73 or 108 day-old transgenics, and 79 or 114 day-old controls) were given drinking water containing 25 mM zinc sulfate for 7 d. Upon stimulation by zinc, the plasma oGH concentration of the transgenic mouse rises to over 100 ng/mL from the unstimulated level of about 12 ng/mL, while control mice have 1–3 ng/mL (2,9). After 7 d of zinc supplementation, the animals were killed by cervical dislocation, and the liver and retro-peritoneal fat were quickly removed.

Isolation of microsomes. All processes were carried out under ice-cold conditions. The fresh liver sample, kept in ice-cold saline, was lightly blotted with a paper towel, weighed, and transferred to a Potter-Elvehjem homogenizer. After adding five times sample weight of homogenizing buffer (pH 7.5 at 4°C, 10 mM tris-HCl, 0.25 M sucrose), the liver was homogenized with ten up–down strokes. Five mL of the homogenate was centrifuged at 12,000 × *g* for 30 min to remove debris and the mitochondrial fraction. The remaining portion of the homogenate was saved and stored at −70°C for fatty acid analysis. The supernatant was then centrifuged at 110,000 × *g* for 60 min. The resulting microsome pellet was resuspended by adding 3 mL of incubation buffer (pH 7.4, 0.04 M phosphate buffer, 0.25 M sucrose, 0.15 M KCl, 0.70 mM *N*-acetylcysteine), and stored at −70°C until enzyme analysis.

Desaturase assay. The detailed assay for Δ6-desaturase activity has been described elsewhere (10). Desaturase activity was assayed in the incubation buffer with the final concentrations of 33 μM ^{14}C-linoleic acid as substrate, 1.3 mM ATP, 5 mM $MgCl_2$, 60 μM CoA, 870 μM NADH, 40 mM NaF, and 330 μM nicotinamide. The reaction was started by adding microsomes, and continued in a flat-bottom vial (25 mm diameter) in a Gyrotory Water Bath Shaker (New Brunswick, Edison, NJ) at 35°C and 110 cycles/min for 15 and 30 min. The reaction was stopped by adding 2 mL saponifying reagent (2 M KOH in methanol). All subsequent steps were performed under nitrogen with added butylated hydroxytoluene to prevent peroxidation of fatty acids. The acyl-CoA was saponified, acidified, and extracted by petroleum ether. Fatty acids were methylated by 5% acetyl chloride in methanol. Fatty acid methyl esters were separated on silver nitrate-impregnated thin-layer silica gel plates using a petroleum ether/diethyl ether solvent system. Separated substrate and product were eluted from the silica by xylene-based scintillation cocktail, and the radioactivity counted. Microsomal and cytosolic proteins were analyzed by a modified Lowry method (11).

Liver and adipose fatty acid analysis. The detailed procedure has been reported elsewhere (12). Briefly, tissue lipids were extracted with chloroform/methanol (2:1, vol/vol), and the extracted lipids were separated by thin-layer chromatography into three fractions, PL, triglyceride (TG), and cholesteryl esters (CE). Fatty acids from each lipid fraction were converted to methyl esters, and the relative quantities were determined by capillary gas chromatography. The absolute amount of each lipid fraction was measured by comparison to the respective heptadecanoate internal standard that was added at the extraction step. Statistical analysis consisted of two-tailed *t*-tests of the means of the two groups, as indicated in Tables 1–3.

RESULTS

As shown in Table 1, after 7 d of zinc feeding the body and liver weights of the transgenic mice compared to controls were 21 and 44% higher, respectively. Consequently, the liver/body weight ratio was higher in transgenic mice. Liver protein concentration did not differ between the two groups in either the homogenate or microsome fraction. Among liver lipids, the TG content was increased more than 3-fold, whereas PL and CE were unchanged. The activity of hepatic Δ6-desaturase for the transgenic mice was elevated more than 2.5-fold when the activity was expressed per mg microsomal protein and per gram liver. The difference became 3-fold when liver Δ6-desaturase activity was adjusted for body size (Table 1).

Table 2 shows the fatty acid profiles of the liver lipid fractions. The substrates of Δ6-desaturase, linoleic acid (18:2n-6), and α-linolenic acid (18:3n-3), were decreased in all three

TABLE 1
Body Weight, Liver Profile, and Δ6-Desaturase Activity[a]

	Control	Transgenic	Transgenic/ control
Body weight (g)	20.6 ± 0.7	24.9 ± 0.4[a]	1.21
Liver weight (g)	1.11 ± 0.03	1.59 ± 0.04[b]	1.44
Liver/body (w/w%)	5.38 ± 0.15	6.37 ± 0.16[c]	1.18
Liver proteins (mg/g liver)			
Homogenate	168 ± 3.7	163 ± 4.0	0.97
Microsome	15.5 ± 0.45	14.7 ± 0.53	0.95
Liver lipids (mg/g liver)			
Phospholipids	17.6 ± 0.43	18.5 ± 0.23	1.05
Triglycerides	5.7 ± 0.7	19.7 ± 6.6[d]	3.46
Cholesteryl esters	1.29 ± 0.22	1.46 ± 0.11	1.13
Δ6-Desaturase activity			
per mg protein (pmol/min)	80 ± 12	212 ± 34[c]	2.64
per g liver (nmol/min)	1.23 ± 0.21	3.14 ± 0.36[c]	2.55
per g body weight (pmol/min)	66 ± 11	197 ± 19[b]	3.00

[a]Weight difference due in part to slight difference in initial body weights associated with slight difference in group mean age. Mean ± SEM, n = 6 for each group except for Δ6-desaturase activity (n = 4 each). Means of the two groups are significantly different by a two-tailed test: [b]$P < 0.001$, [c]$P < 0.01$, [d]$P < 0.05$.

TABLE 2
Fatty Acid Profile of Liver Lipid Fractions[a]

	Liver phospholipids		Liver triglycerides		Liver cholesteryl esters	
	Control	Transgenic	Control	Transgenic	Control	Transgenic
16:0	21.47 ± 0.29	20.67 ± 0.44	22.49 ± 0.48	22.66 ± 0.72	25.69 ± 2.29	44.02 ± 3.73[b]
18:0	17.14 ± 0.25	22.05 ± 0.32[c]	3.31 ± 0.15	3.92 ± 0.41	7.51 ± 0.46	6.41 ± 0.17
18:1n-9	7.46 ± 0.26	4.05 ± 0.20[c]	36.39 ± 0.95	40.28 ± 1.19[d]	18.65 ± 1.24	7.53 ± 1.25[c]
18:2n-6	16.35 ± 0.19	9.36 ± 0.26[c]	19.06 ± 0.64	16.29 ± 0.74[d]	9.36 ± 0.73	7.19 ± 0.72
18:3n-6	0.24 ± 0.02	0.30 ± 0.02	0.35 ± 0.06	0.68 ± 0.03[c]	0.17 ± 0.17	0.24 ± 0.05
18:3n-3	0.08 ± 0.00	0.04 ± 0.00[c]	0.66 ± 0.08	0.41 ± 0.02[d]	1.04 ± 0.15	0.49 ± 0.05[b]
20:3n-9	0.11 ± 0.01	0.09 ± 0.01	0.13 ± 0.02	0.29 ± 0.05[b]	0.29 ± 0.09	0.20 ± 0.06
20:3n-6	2.09 ± 0.11	0.77 ± 0.04[c]	0.65 ± 0.04	0.48 ± 0.05[d]	0.53 ± 0.25	0.53 ± 0.10
20:4n-6	11.22 ± 0.27	17.36 ± 0.67[c]	1.15 ± 0.05	1.63 ± 0.28	3.41 ± 1.09	7.25 ± 0.98[d]
20:5n-3	1.38 ± 0.06	0.75 ± 0.06[c]	0.80 ± 0.09	0.42 ± 0.05[b]	3.31 ± 0.46	2.10 ± 0.20
22:6n-3	16.33 ± 0.32	19.84 ± 0.45[c]	6.74 ± 0.20	3.10 ± 0.54[c]	2.14 ± 0.31	4.00 ± 0.98

[a]Fatty acids expressed as weight %, mean ± SEM (n = 6 except n = 5 for cholesteryl esters of the control group). Means of the two groups significantly different by a two-tailed test: [b]$P < 0.01$, [c]$P < 0.001$, [d]$P < 0.05$.

lipid fractions of transgenic mice in comparison with the control group, although the reduction in linoleic acid in the CE did not reach statistical significance. On the other hand, 20:4n-6, the major product of the desaturase/elongase pathway for essential fatty acids, was elevated in all three liver lipid fractions of transgenic mice although it did not reach statistical significance in the TG fraction. The final product of n-3 essential fatty acids, 22:6n-3, was increased in the liver PL of transgenic mice, but decreased in the liver TG. Another final product of the same pathway, Mead acid (20:3n-9), was increased in the liver TG fraction of transgenic mice. Among the intermediates of the desaturase/elongase pathway, 20:3n-6 and 20:5n-3 were decreased in both PL and TG fractions in liver of transgenic mice, but 18:3n-6 was increased in the TG fraction (Table 2).

Saturated and monounsaturated fatty acids showed variable responses among liver lipid fractions. Palmitic acid (16:0) was unchanged in the liver PL and TG fractions between the transgenic and control groups, but was markedly increased in the CE fraction of transgenic mice. Stearic acid (18:0) was increased only in the liver PL of transgenic mice. Transgenic mice showed an increase in oleic acid (18:1n-9) in liver TG, but it was decreased in liver PL and CE (Table 2).

Table 3 reports the fatty acid content of adipose PL and TG. In adipose PL from transgenic mice, there was an increase in palmitic acid at the expense of significant decreases in 20:4n-6 and 22:6n-3 (which were relatively increased in the liver PL of transgenic mice). The adipose TG fatty acid composition was relatively unchanged, with the only significant differences being decreased 18:0 and increased 20:4n-6 in the transgenic mice.

TABLE 3
Fatty Acid Profile of Adipose Phospholipids and Triglycerides[a]

	Adipose phospholipids		Adipose triglycerides	
	Control	Transgenic	Control	Transgenic
16:0	28.70 ± 1.23	35.40 ± 1.36[b]	21.93 ± 0.33	21.80 ± 0.61
18:0	15.05 ± 2.29	15.80 ± 3.43	6.44 ± 0.14	3.68 ± 0.15[c]
18:1n-9	12.44 ± 1.96	10.11 ± 2.55	40.40 ± 0.33	40.49 ± 0.78
18:2n-6	9.52 ± 0.81	4.12 ± 0.36[c]	18.69 ± 0.24	19.16 ± 0.16
18:3n-6	0.25 ± 0.07	0.32 ± 0.05	0.21 ± 0.01	0.19 ± 0.01
18:3n-3	0.17 ± 0.04	0.18 ± 0.02	0.76 ± 0.02	0.83 ± 0.03
20:3n-9	0.17 ± 0.06	0.24 ± 0.08	0.04 ± 0.01	0.04 ± 0.00
20:3n-6	0.36 ± 0.05	0.13 ± 0.01[b]	0.18 ± 0.01	0.14 ± 0.01
20:4n-6	2.91 ± 0.23	1.71 ± 0.20[b]	0.20 ± 0.01	0.28 ± 0.02[d]
20:5n-3	0.52 ± 0.05	0.21 ± 0.05[b]	0.14 ± 0.01	0.17 ± 0.03
22:6n-3	2.90 ± 0.18	1.86 ± 0.19[b]	0.70 ± 0.04	0.65 ± 0.08

[a]Fatty acids expressed as weight %, mean ± SEM (n = 5 for phospholipids and n = 6 for triglycerides). Means of the two groups significantly different by a two-tailed test: [b]$P < 0.01$, [c]$P < 0.001$, [d]$P < 0.05$.

DISCUSSION

The Δ6-desaturase, the gate keeper enzyme of the Δ6 desaturase/elongase/Δ5 desaturase pathway for 20:4n-6 synthesis, is subject to control by various hormones including steroid hormones, epinephrine, glucagon, and insulin. Most of these hormones suppress Δ6-desaturase activity, although insulin may stimulate the enzyme (13). In this study, we have demonstrated a marked increase in Δ6-desaturase activity in the transgenic mice following oGH induction for 1 wk with 25 mM zinc (Table 1). The increased Δ6-desaturase activity in the transgenic mice is reflected in the alteration of the fatty acid composition in liver PL, TG, and CE fractions, which showed decreased proportions of substrates (18:2n-6 and 18:3n-3) and increased final products (20:4n-6, 22:6n-3, and 20:3n-9) except for 22:6n-3 in the TG fraction (Table 2). However, as the total TG in the livers of the transgenic mice was increased approximately 3-fold, the absolute amount of 22:6n-3 in the TG fraction was actually increased despite its lower proportion.

Maddaiah and Clejan (5) reported that the 20:4n-6/18:2n-6 ratio in PL from liver microsomes and mitochondria of hypophysectomized rats was significantly elevated as early as 4 h after a single GH injection. This rapid change in PL fatty acid composition suggests that GH may also affect turnover and distribution of PL fatty acid *via* altering activities of enzymes such as phospholipases and lysophospholipid acyl transferases. The resultant effects of GH on adipose tissue

could be multifactorial. As GH has been shown to stimulate differentiation of preadipocytes to adipocytes both *in vitro* (14,15), and *in vivo* (16), it may play a role in adipose tissue development. Acute administration (20–30 min) of GH stimulated glucose uptake by adipocytes *via* the same mechanism as insulin (17), and inhibited epinephrine-stimulated lipolysis (18). Both of these effects would tend to increase adiposity. The other side of GH is its inhibitory effect on TG accumulation by adipose tissue. In humans, GH administration reduced fat and increased lean body mass of GH-deficient adults (19). GH also increased fat oxidation when administered to normal men (20). Contrary to its acute effect, chronic administration of GH increased hormone-sensitive lipase activity and glycerol release by cultured adipocytes (18). Schoenle *et al.* (21) reported that basal (no insulin stimulation) glucose transport was elevated to the insulin-stimulated level in adipocytes from GH-deficient patients, but it was suppressed to the normal level by GH treatment. Taken in combination, these observations point to the likelihood that chronic administration of GH to mature animals has an antilipogenic effect on adipose tissue.

The role of membrane 20:4n-6 in the function of adipose tissue has not been well defined. Gaillard *et al.* (22) showed that 20:4n-6 was required for the differentiation of a preadipocyte cell line, and this arachidonic acid effect appears to be mediated by prostaglandins (23). This observation has been reinforced by Serrero *et al.* (24), who observed that eicosanoids derived from arachidonate are involved in adipocyte differentiation. Pelikanova *et al.* (25) reported a positive correlation between insulin action and plasma 20:4n-6, and Borkman *et al.* (26) showed that the 20:4n-6 level in human muscle PL correlated with insulin sensitivity. Based on these studies, it is a logical extension that 20:4n-6 modulates insulin sensitivity in adipose tissue as well.

Altered availability of 20:4n-6 has also been documented to affect multiple other cellular functions, such as gene transcription of fatty acid synthase (27) and heat shock proteins (28), and it participates in the regulation of insulin secretion in part by inactivating protein kinase C (29). Arachidonate and other highly unsaturated fatty acids also modulate many processes affecting immune function (30,31).

Among the lipid fractions examined in this study, the adipose PL was the only fraction which showed a lower proportion of 20:4n-6 in the transgenic mice compared with the controls. This indicates that the fatty acid profile of adipose PL was not passively reflecting the production of highly unsaturated essential fatty acids in liver, but was subject to altered distribution by GH. The same response was seen with 22:6n-3, and these changes were parallel to those reported by Maddaiah and Clejan (5). If reduced, 20:4n-6 is associated with insulin resistance; then our observation of the reduced arachidonic acid in adipose PL could result in increased adipose lipolysis.

In summary, this study demonstrated that induction of an oGH transgene increased Δ6-desaturase activity and its resulting products including 20:4n-6 in liver lipids and adipose TG, but decreased 20:4n-6 and 22:6n-3 in adipose PL. The mechanism through which this GH effect alters adipose PL fatty acid composition is unknown at present. This complex alteration in the production and distribution of 20:4n-6 between tissues may be related to the effect of GH on energy balance, insulin sensitivity, immunity, and adipocyte functions.

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An *in vivo* ^{13}C Magnetic Resonance Spectroscopic Study of the Relationship Between Diet and Adipose Tissue Composition

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ABSTRACT: ^{13}C magnetic resonance spectroscopy (MRS) is a noninvasive technique used in the study of lipids. We applied ^{13}C MRS to assess the effects of long-term dietary variation on adipose tissue composition in humans. *In vivo* ^{13}C MRS was used to analyze the fatty acid composition of adipose tissue in 88 healthy volunteers with significantly different diets (38 vegans, 11 vegetarians, and 39 omnivores) assessed by analysis of dietary records. Results were compared with the serum lipid profile. ^{13}C MRS revealed clear differences in the adipose tissue composition of vegans, which contained more unsaturated ($P < 0.01$) and fewer saturated fatty acids ($P < 0.01$) compared with omnivores and vegetarians. The vegan subjects had a significantly lower intake of saturated fatty acids and higher intake of polyunsaturated fatty acids than either the omnivore or the vegetarian groups ($P < 0.01$). These findings were associated with significantly lower levels of serum total cholesterol and low density lipoprotein-cholesterol in the vegan group compared with the omnivores. Our results demonstrate the use of ^{13}C MRS for the noninvasive study of adipose tissue composition and its application to the study of the interaction between long-term dietary and metabolic risk factors in humans.
Lipids 31, 145–151 (1996).

Coronary heart disease (CHD) remains a leading cause of death in the western world. Plasma cholesterol concentrations, central body fat distribution, and the dietary saturated fat content are recognized risk factors for CHD (1–4). Dietary unsaturated fatty acids have been shown to lower the concentration of plasma cholesterol (5,6) and are thought to reduce the risk of CHD. It has also been shown that adipose tissue fatty acids alter plasma lipid profiles and are correlated with potential risk of CHD (7). Screening subjects considered at risk of CHD is routinely performed by analyzing serum cholesterol and the lipoprotein profile (8–11). However, less attention has been directed to the contribution from adipose tissue composition.

Dietary fat is a major factor determining the fatty acid composition of adipose tissue, and the half-life of fatty acids in adipose tissue is around 600 d. The fatty acid profile of adipose tissue is therefore thought to reflect long-term dietary intake (12) and has been suggested as an alternative index of the habitual dietary fatty acid intake over the previous two to three years. Previously, studies of the fatty acid composition of adipose tissue from different regions of the body have been limited to gas–liquid chromatography (GLC) analysis of needle fat biopsies (13–21) or postmortem fat samples (13,22). Routine measurement of lipid composition by repeated biopsy in both healthy volunteers and patients invokes both ethical and practical difficulties. This has limited the number of serial studies looking at long-term dietary effects on adipose tissue composition. A noninvasive method of determining lipid composition is therefore required.

Magnetic resonance spectroscopy (MRS) is a technique that allows human biochemistry to be studied noninvasively. ^{13}C MRS has been successfully applied to the study of lipids *in vivo*, both in humans (23–25) and in animals (26–28). Although specific fatty acids cannot be assessed by ^{13}C MRS, measurements of the major classes of unsaturated and saturated fatty acids can be made (24). Furthermore, good correlation has been reported between GLC and ^{13}C MRS in both humans (24) and in animals (27,28). Animal studies suggest that *in vivo* ^{13}C MRS can detect changes in adipose tissue lipid composition arising from dietary manipulation (27,28). Beckmann (23) used ^{13}C MRS to noninvasively study the adipose tissue composition of human subjects on a standard and low-fat diet. MR spectra of adipose tissue were shown to reflect the subjects' diet (23).

In this study we assessed the use of *in vivo* ^{13}C MR spectroscopy to investigate the long-term effects of different diets on adipose tissue composition in healthy human volunteers. The results from this study are discussed in relation to dietary analysis and the lipid metabolic risk profile.

MATERIALS AND METHODS

Volunteer assessment. Written informed consent was obtained from all volunteers. Permission for this study was obtained from the ethics Committee of the Royal Postgraduate Med-

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Abbreviations: CHD, coronary heart disease; GLC, gas–liquid chromatography; HDL-c, high density lipoprotein-cholesterol; LDL-c, low density lipoprotein-cholesterol; MRS, magnetic resonance spectroscopy.

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ical School, Hammersmith Hospital, London (REC 4096/93). Healthy volunteers were studied and were screened to exclude chronic illness, including diabetes and liver disease. Eighty-eight subjects were examined in total, and were divided into three age and sex-matched groups according to their dietary history: 38 vegans: 20–69 yr (35.1 ± 14.4) 21 males, 17 females; 39 omnivores: 20–74 yr (43.8 ± 16.9) 20 males, 19 females; and 11 vegetarians: 19–62 yr (34.2 ± 13.2) 6 males, 5 females.

The vegan diet excludes all forms of animal products. Ovo-lacto vegetarians do not consume meat or fish, but their diet does include dairy products. Volunteers were weight stable and had maintained their current diet for a minimum of three years.

Biochemical analysis. Fasting blood samples were drawn from each volunteer for serum lipid analysis, including total cholesterol, low density lipoprotein-cholesterol (LDL-c), high density lipoprotein-cholesterol (HDL-c), and triglycerides. Serum total cholesterol, HDL-c, and serum total triglyceride concentrations were determined enzymatically using standard methods (Boehringer, Mannheim, Germany). LDL-c was calculated from these values using the Friedewald equation. Repeated blood samples were obtained from six volunteers over a six-week period to assess the degree of variation in individual serum cholesterol levels. Screening tests also included full blood count and biochemical profile, including serum glucose, liver function tests, urea, and electrolyte estimates.

Dietary assessment. Each volunteer completed a seven-day dietary diary within one week of their *in vivo* ^{13}C MRS examination, using methodology validated by Bingham (29). Subjects were given a standard booklet containing written instruction on prospectively recording their food intake, and this written information was backed up by verbal advice. The diaries were then returned by mail, and the volunteers were contacted by telephone if clarification of intake was needed. Dietary analysis was carried out using a standard database (Microdiet, Salford University, Salford, United Kingdom) containing all data from current food tables (30). Validation was carried out by comparing the agreement (31) between estimated energy intakes from the diet diaries to estimate energy requirement from a standard formula (32). For subjects in energy balance, there should be good agreement between the two variables. The mean standard error of differences was −84 kcal, suggesting a slight underreporting with reasonable standard deviation for this type of comparison of ± 254 kcal. All volunteers included in this study fully completed their dietary records.

Anthropometric measurements. Anthropometric assessment was obtained on each subject by a trained observer. This included measurement of weight, height, waist, and hip circumference, and skinfold thickness from triceps, biceps, subscapular, and suprailiac regions. Body mass index, percentage body fat, and total body fat were calculated for each individual using standard methods (33).

^{13}C MRS. ^{13}C MR spectra were obtained from the adipose tissue of all 88 healthy volunteers. *In vivo* ^{13}C MR spectra were acquired on a 1.5 T Picker prototype system (Picker International, Cleveland, OH) using a 20-cm saddle transmitter coil and a 14-cm receiver coil, placed orthogonally to the left thigh of each subject. Fully relaxed coupled ^{13}C spectra were acquired using a 90° pulse, with eight averages and a repetition time of 30 s. The average time for each examination was 15 min.

Spectra were zero-filled to 4 K and Fourier transformed with no line broadening. Peaks were integrated using a curve-fitting computer model (NMR1;® New Methods Res., Inc., East Syracuse, NY). Six volunteers had ^{13}C MR spectra collected from various parts of their bodies, including thigh, waist, and buttocks, to determine possible regional variation in adipose tissue composition.

MRS data analysis. (i) Total fat analysis by ^{13}C MRS (Method A). Total unsaturated and saturated fatty acids were calculated (Table 1) as previously described by Moonen *et al.* (24), briefly: (see Fig. 1):

$$\% \text{ polyunsaturated} = \tfrac{1}{2}(I_{2b} + I_{3b})/I_1 \times 100 \quad [1]$$

$$\% \text{ monounsaturated} = \tfrac{1}{2}[(I_{2a} + I_{3a}) - (I_{2b} + I_{3b})]/I_1 \times 100 \quad [2]$$

$$\% \text{ saturated} = 100 - (\% \text{ polyunsaturated} + \% \text{ monounsaturated}) \quad [3]$$

This method allows direct comparison with literature values obtained by GLC (23,24). However, this method cannot be used accurately to determine proportions of monounsaturated and polyunsaturated fat present when fatty acids with more than two double bonds are present. The method of percentage carbon analysis as suggested by Barnard (27) was therefore also used.

(ii) Percentage carbon analysis by ^{13}C MRS (Method B). Most MRS methods for calculating polyunsaturated fatty acid content assume linoleic acid (18:2n-6) to be the principal polyunsaturated fatty acid present in human adipose tissue (85–90% in adults) (21,24). Here only two carbons would contribute to the resonances at 133.4 and 124.2 ppm in the coupled ^{13}C MR spectrum. If fatty acids with more than two double bonds are present, such as linolenic acid (18:3n-3) or arachidonic acid (20:4n-6), these assumptions will introduce large errors into the calculations, a particular problem in neonates (34–36) and vegans. We therefore calculated the percentage of total carbons (Table 2) in saturated or unsatu-

TABLE 1
Percentage Fat Determined by ^{13}C Nuclear Magnetic Resonance Spectroscopy (Method A)[a]

Diet type	% Total unsaturated	% Total saturated
Vegan	86.5 ± 0.9[b]	13.5 ± 0.9[b]
Omnivore	76.1 ± 0.6	23.9 ± 0.6
Vegetarian	78.9 ± 1.6[c]	21.1 ± 1.6[c]

[a]Mean ± SEM. Significant difference between vegans and omnivore controls, [b]$P < 0.01$; significant difference between vegans and vegetarians, [c]$P < 0.01$; no significant differences were found between vegetarians and omnivore controls.

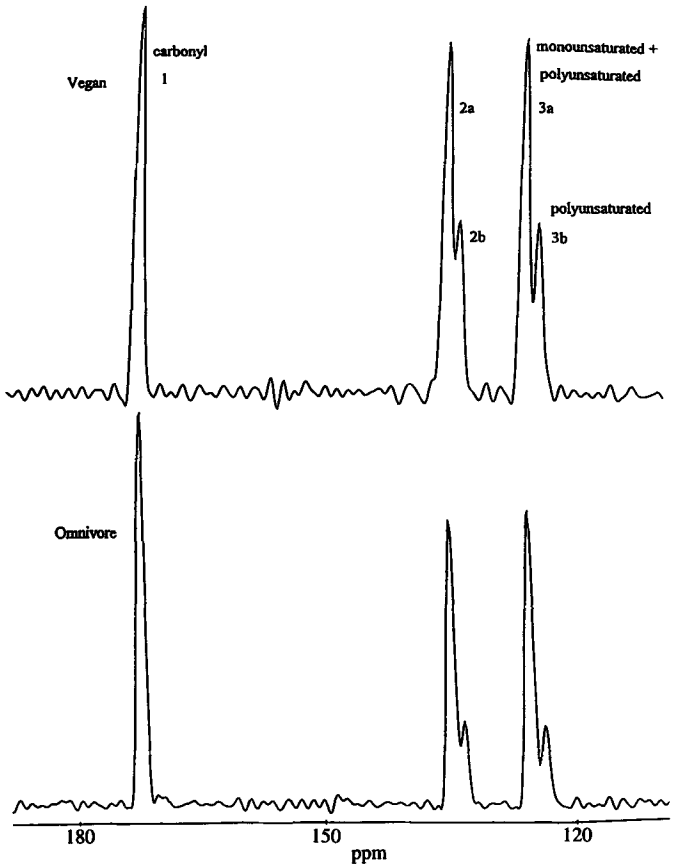


FIG. 1. ^{13}C Magnetic resonance spectra of human adipose tissue from a vegan and an omnivore expanded to show the unsaturated region (repetition time 30 s). Assignments: 1, carbonyl carbons (-C=O) 172.1 ppm; 2a and 3a, carbons in the monounsaturated and polyunsaturated position (-CH=CH- and -CH=CH-CH2-CH=CH-) 134.9 and 125.4 ppm; and 2b and 3b, polyunsaturated carbons only (-CH=CH-CH_2-CH=CH-) 133.4 and 124.2 ppm.

rated position in the fatty acid chain. This method is based on absolute data and makes no assumptions about the composition of the tissue under study (27).

Percentages of carbons in saturated and unsaturated position in the fatty acid chain were calculated from integrals of three regions of the ^{13}C MR spectrum—carbonyl at 165–175 ppm; unsaturated fatty acid carbons at 120–140 ppm; and saturated fatty acid carbons at 10–45 ppm.

$$\text{total carbon in fatty acid chain (A)} = I_{carbonyl} + I_{unsat} + I_{sat} \quad [4]$$

$$\text{percentage carbon in unsaturated position (B)} = (I_{unsat}/A) \times 100 \quad [5]$$

$$\text{percentage carbon in saturated position} = 100 - B \quad [6]$$

The unsaturated region has two pairs of resonances: (i) the larger peaks (I_{m+p}) 134.9 and 125.4 ppm from carbons in the monounsaturated and polyunsaturated position (-CH=CH= and -CH=CH=CH_2-CH=CH-) and (ii) the other peaks (I_p) 133.4 and 124.2 ppm from polyunsaturated fatty acid carbons (-CH=CH-CH_2-CH=CH-). Using peak height measurements or integrals of these peaks (obtained using a curve-fitting algorithm), the proportion of fatty acid carbons that were polyunsaturated or monounsaturated could be determined and used in the equations below:

$$\text{percentage carbon in a poly position} = I_p/(I_{m+p} + I_p) \times \% \text{ unsaturated (B)} \quad [7]$$

$$\text{percentage carbon in a mono position} = \% \text{ unsaturated} - \% \text{ polyunsaturated} \quad [8]$$

Statistical analysis. Statistical analysis was performed using Student's unpaired *t*-test. Correlation coefficients were calculated using the Pearson's test. All results presented are expressed as mean ± SEM.

RESULTS

Biochemical analysis. Results of the serum lipid analysis are shown in Table 3. Significantly lower concentrations of total cholesterol (4.41 ± 0.24 vs. 5.31 ± 0.24 mmol/L, $P < 0.01$) and LDL-c (2.46 ± 0.20 vs. 3.41 ± 0.23 mmol/L, $P < 0.05$) were observed in the vegan group compared with the omnivore population. No significant differences of triglycerides or HDL-c were observed in the vegans compared with the omnivores. The vegetarian group had significantly lower concentrations of LDL-c (2.65 ± 0.16 vs. 3.41 ± 0.23 mmol/L, $P < 0.05$) than the omnivores. However, no significant differences were observed between the lipid profiles of vegan and vegetarian groups.

TABLE 2
Percentage Carbons in Saturated or Unsaturated Positions in the Fatty Acid Chain Calculated by ^{13}C MRS (Method B)[a]

Diet type	% Saturated	% Total unsaturated	% Polyunsaturated[b]	% Monounsaturated[c]
Vegan	87.9 ± 0.2[d]	11.4 ± 0.5[e]	3.5 ± 0.1[d]	8.3 ± 0.3
Omnivore	89.8 ± 0.2	10.2 ± 0.2	2.3 ± 0.01	7.9 ± 0.1
Vegetarian	89.5 ± 0.3[g]	10.5 ± 0.3	2.6 ± 0.1[f]	7.9 ± 0.3

[a]Mean ± SEM.

[b]Polyunsaturated fatty acids and refers to carbons in -CH=CH-CH_2-CH=CH- position.

[c]Monounsaturated fatty acids and refers to carbon in -CH=CH and -CH=CH=CH_2-CH=CH-position.

[d]$P < 0.01$, [e]$P < 0.02$ difference between vegans and omnivore controls. [f]$P < 0.01$, [g]$P < 0.02$ difference between vegans and vegetarians. No significant differences were found between vegetarians and omnivore controls.

TABLE 3
Serum Lipid Profiles[a]

Diet	Cholesterol	LDL-c[b]	HDL-c[c]	Triglyceride
Vegan	4.41 ± 0.24[d]	2.46 ± 0.20[d]	1.35 ± 0.08	0.94 ± 0.09
Omnivore	5.31 ± 0.24	3.41 ± 0.23	1.44 ± 0.06	1.04 ± 0.09
Vegetarian	4.57 ± 0.38	2.65 ± 0.16[e]	1.38 ± 0.07	1.02 ± 0.12

[a]Mean ± SEM (mmol/L).
[b]LDL-c = low density lipoprotein-cholesterol.
[c]HDL-c = high density lipoprotein-cholesterol.
[d]Significant difference between vegans and omnivore controls; $P < 0.05$. No significant differences were found between vegans and vegetarians.

Dietary assessment. Dietary assessment of seven-day food records (Table 4) showed no significant differences in total calorific intake or the percentage total dietary fat intake between the three groups. As a whole group, the expected calorie intake was 2316 ± 54 kcal compared with 2231 ± 74 kcal reported calories.

The vegan population has a significantly higher intake of unsaturated fatty acids, particularly polyunsaturated fatty acids (37.9 ± 1.6 vs. 20.5 ± 1.2%, $P < 0.01$). They had a correspondingly lower intake of saturated fatty acids (22.3 ± 1.0 vs. 42.1 ± 1.2%, $P < 0.01$) in their diet than the omnivore groups. Vegans also have a significantly lower intake of saturated fatty acids than vegetarians (22.3 ± 1.0 vs. 41.7 ± 3.5%, $P < 0.01$). No significant differences in dietary fat content and composition were found between omnivores and vegetarians.

The vegan group also had a significantly higher dietary intake of carbohydrates than either omnivore or vegetarian groups (vegans 331.7 ± 23.2 g, omnivores 243.1 ± 9.4 g, vegetarians 230.8 ± 17.5 g, $P < 0.01$). There was no significant difference in carbohydrate intake between omnivores and vegetarians.

Anthropometric measurements. No significant differences were observed in the total percentage body fat present in either vegan, vegetarian, or omnivore groups. However, the vegan group had a lower body mass index (21.8 ± 0.5 vs. 23.6 ± 0.5 kg/m^2, $P < 0.05$) than the omnivore population (Table 5).

^{13}C MRS. Representative ^{13}C proton-coupled MR spectra obtained from the human thigh *in vivo* are shown in Figure 2. Resonances observed in these spectra arise solely from triglyceride molecules from adipose tissue (24). The peaks arise from the carbonyl, unsaturated, and saturated regions of the fatty acid chains and from the glycerol backbone. The unsaturated region is composed of resonances from monounsaturated fatty acids and polyunsaturated fatty acids. No regional variations were found in the spectra acquired from different areas of the body for individual subjects.

Differences between dietary groups. Expansions of the

TABLE 4
Results of Seven-Day Dietary Diary Analysis[a]

Diet type	% Fat[b]	% Saturated	% Polyunsaturated	% Monounsaturated
Vegan	39.5 ± 1.6	22.4 ± 1.0[c]	37.9 ± 1.6[c]	39.7 ± 1.5
Omnivore	38.2 ± 1.2	42.1 ± 1.2	20.5 ± 1.2	37.4 ± 0.8
Vegetarian	38.4 ± 3.6	41.7 ± 3.5[d]	20.2 ± 3.3[d]	38.1 ± 1.9

[a]Mean ± SEM.
[b]Fat = percentage total dietary fat intake.
[c]Significant difference between vegans and omnivore controls. $P < 0.01$; significant difference between vegans and vegetarians; [d]$P < 0.01$; no significant differences were found between vegetarians and omnivore controls.

TABLE 5
Anthropometric Measurements[a]

Group	BMI[b] (kg/m^2)	% Body fat	Total body fat (kg)
Vegan	21.8 ± 0.5[c]	21.9 ± 1.5	14.0 ± 1.1
Omnivore	23.6 ± 0.5	24.0 ± 1.6	17.4 ± 1.5
Vegetarian	22.6 ± 1.4	22.5 ± 2.5	15.3 ± 1.8

[a]Mean ± SEM.
[b]BMI = body mass index.
[c]Significant difference between vegans and omnivore controls $P < 0.05$. No significant differences were found between vegans and vegetarians or vegetarians and omnivore controls.

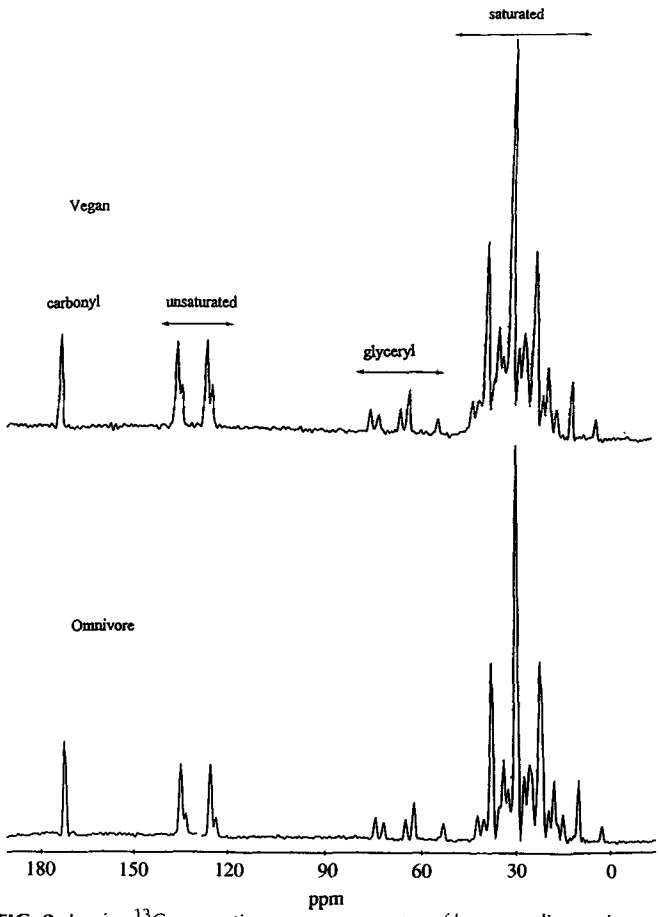


FIG. 2. *In vivo* ^{13}C magnetic resonance spectra of human adipose tissue from a vegan and an omnivore (repetition time 30 s). Resonances for the carbonyl, unsaturated, and saturated regions of fatty acid chains and from the glycerol backbone of the triglycerides may be observed. Assignments: carbonyl 170–175 ppm; monounsaturated and polyunsaturated fatty acid carbons 120–140 ppm; glycerol backbone 60–70 ppm; saturated fatty acid carbons 10–40 ppm.

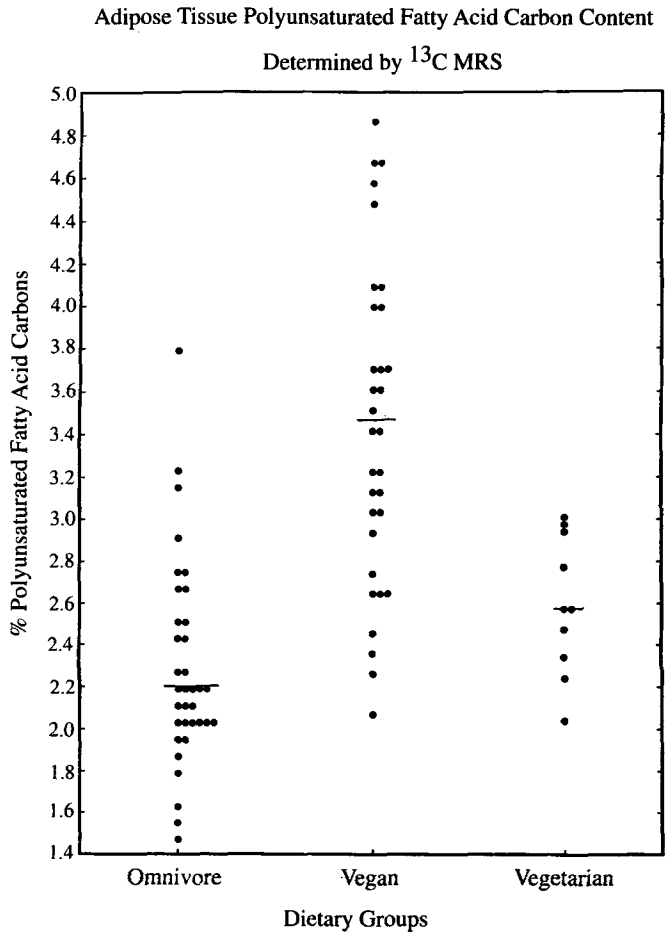


FIG. 3. Adipose tissue polyunsaturated fatty acid carbon content determined by ^{13}C magnetic resonance spectroscopy (MRS) for omnivore, vegan, and vegetarian dietary groups (Method B). This figure illustrates the increased mean polyunsaturated fatty acid content (—) in the vegan group, which showed a wide range of values and a marked increase in some subjects (maximum value 4.9%).

olefinic region of the ^{13}C MR spectra obtained from an omnivore and a vegan volunteer are shown in Figure 1. Significant differences in the relative intensity of resonances arising from unsaturated fatty acids can be observed. The vegan subjects have significantly more total unsaturated fat (86.5 ± 0.9%) than either vegetarian (78.9 ± 1.6%, $P < 0.01$) or omnivore groups (76.1 ± 0.6%, $P < 0.01$) (Method A) (Table 1).

Similarly, when the fatty acid content of adipose tissue is expressed as percentage carbon (Method B), these differences in saturated and unsaturated fatty acid carbons can again be observed (Table 2). Furthermore, this method allows us to assess the relative content of polyunsaturated and monounsaturated lipids without making assumptions as to the type of fatty acids present. Adipose tissue of vegan subjects had significantly more polyunsaturated fatty acid carbons (3.5 ± 0.1%) than either omnivore (2.3 ± 0.01%, $P < 0.01$) or vegetarians (2.6 ± 0.1%, $P < 0.01$) (Fig. 3). The relative content of monounsaturated fatty acids was similar in all groups. No significant differences in adipose tissue composition were found between the vegetarian and omnivore groups.

Correlation between MRS and dietary analysis. Significant correlations were found comparing dietary saturated fat intake with MRS measurement of both saturated ($r = 0.55$, $P < 0.01$) and polyunsaturated ($r = -0.64$, $P < 0.01$) fatty acid content of adipose tissue (Method B). A strong correlation was also found between the polyunsaturated/saturated ratio of dietary fat intake and the MRS measurement of polyunsaturated fatty acids in adipose tissue ($r = 0.61$, $P < 0.01$) (Method B). No correlations were found to exist between serum lipid levels and dietary intake, or between serum lipid levels and fatty acid content of adipose tissue, as determined by ^{13}C MRS.

DISCUSSION

In this study we have demonstrated that *in vivo* ^{13}C MRS can be used to assess the effects of diet on adipose tissue composition. Measurement of individual fatty acids, using natural abundance ^{13}C MRS, is not possible because of the limited

resolution of *in vivo* spectroscopy in humans. However, our results confirm that ^{13}C MRS offers a unique opportunity to noninvasively study dietary influences on adipose tissue lipid composition, allowing repeated measurements of the proportion of saturated and unsaturated fatty acids in adipose tissue (23,24). Significant differences were found in the relative levels of polyunsaturated and saturated fatty acids in the subcutaneous tissue of vegan volunteers compared with those on an omnivore diet.

Good agreement has previously been reported when directly comparing MRS and GLC analysis of adipose tissue composition (24,27,28). Overall reported values for the percentage of unsaturated fatty acids found in adipose tissue biopsies by GLC vary considerably (60–80%) (13–22). Subjects in these GLC studies had extremely different fat intake and our study has highlighted the dependence of adipose tissue composition on dietary fat content. Our results from omnivore subjects obtained using ^{13}C MRS were similar to these reported values, although in the upper part of this range (76.1 ± 0.6%). This can be explained by the fact that all three of our diet groups had a higher intake of dietary polyunsaturated fatty acids than in many other subject groups reported in the GLC literature.

Significant correlations between the MRS data and dietary assessment were obtained for saturated and polyunsaturated fatty acids, but not for monounsaturated fatty acids, a finding that agrees with previous studies (37). Dietary assessment of fat intake is dependent on the reliability of the individual dietary records. MRS may give a better indication of the long-term dietary history of a given subject, as it is not sensitive to short-term fluctuations in the amount and composition of the day-to-day diet. An interesting finding was the similarity between the vegetarian and omnivore groups in terms of diet and adipose tissue composition. It would appear that the vegetarians were replacing one form of saturated fat in their diet (meat) for another (dairy products), resulting in very little difference in the overall composition of their fat intake compared with an omnivore diet.

The levels of serum total cholesterol and LDL-c were shown to be significantly different in the vegans compared with omnivores. However, serum lipids were not strongly related to adipose tissue composition. This may arise from the large fluctuation of serum lipid levels (physiological/dietary related) known to occur, compared with the more stable composition of adipose tissue.

In vivo assessment of adipose tissue composition showed marked elevation in the levels of polyunsaturated and monounsaturated fatty acids in healthy subjects on a long-term vegan diet compared with healthy omnivores and vegetarians. This is an important finding, as it is thought that there is an inverse relationship between polyunsaturated fat in adipose tissue and the risk of CHD, though this is not fully understood (5).

In vivo ^{13}C MRS provides a unique opportunity to study lipid metabolism in humans. It provides a noninvasive method to study the fatty acid profile and may therefore become an important tool in large-scale or serial epidemiological studies. Determining the interactions between diet, adipose tissue, and serum lipids is an exciting future prospect.

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Lipoprotein Composition and Serum Cholesterol Ester Fatty Acids in Nonwesternized Melanesians

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ABSTRACT: In this study, the relationships between dietary fat [as measured by serum cholesterol ester fatty acids (CE–FA)], age, smoking, body mass index, and serum lipids were analyzed in 151 subsistence horticulturalists, aged 20–86 yr, from Kitava, Trobriand Islands, Papua New Guinea. Their diet consists of tubers, fruit, coconut, fish, and vegetables with a negligible influence of western food and alcohol. Total fat intake is low [21% of energy (en%)], while saturated fat intake from coconuts is high (17 en%, mainly lauric and myristic acid). In multivariate analysis, 11–43% of the variation of the serum lipoprotein composition was explained by CE–FA, age, and smoking habits. The proportion of CE20:5n-3 explained much of the variation of triglycerides (TG, negative relation) and high density lipoprotein-cholesterol (HDL-C, positive) in both sexes and serum apolipoprotein A1 (ApoA1, positive) in the males. CE16:0 was positively related to TG and negatively related to HDL-C and ApoA1 in both sexes, and in males it related negatively to total cholesterol (TC) and low density lipoprotein-cholesterol (LDL-C). In males, negative relationships were present between CE18:2n-6 and TC and between CE14:0 and serum lipoprotein(a). Smoking was independently associated with lower ApoA1 in both sexes and with lower HDL-C and higher TG, TC, LDL-C, and apolipoprotein B in males. In conclusion, marine n-3 fatty acids and linoleic acid showed the same potentially beneficial relationships with lipoproteins and apolipoproteins as in western populations. The relations of palmitic acid to serum lipids may be explained in terms of endogenous fat synthesis at a low-fat intake, rather than reflecting its relative intake.
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The relations between serum lipoproteins and apolipoproteins and atherothrombotic cardiovascular disease are well established, both within and between westernized populations (1–3), with the possible exception of lipoprotein(a) [Lp(a)] (4). Increased total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), apolipoprotein B (ApoB), triglycerides (TG), and Lp(a) increase the risk, and increased high density lipoprotein-cholesterol (HDL-C) and apolipoprotein A1 (ApoA1) decrease it. Levels of serum lipoproteins and apolipoproteins are influenced by the composition of dietary fatty acids (FA) (5) which, in turn, are at least partly reflected by their relative presence in serum cholesterol esters (CE) (6–17).

In the island of Kitava, Trobriand Islands, Papua New Guinea, we have investigated a population of subsistence horticulturalists in whom stroke and ischemic heart disease (IHD) appear virtually absent despite high smoking rates (18). Diastolic blood pressure, body mass index (BMI; kg/m^2), and triceps skinfolds are very low as compared to healthy subjects from Sweden (where incidence rates of stroke and IHD are high), while systolic blood pressure and LDL-C are only slightly lower and in females only after 60 years of age (19).

The diet in Kitava is peculiar: total fat intake is low but saturated fat intake is high, and most of the dietary fat is derived from coconut and fish (20). The average level of physical activity is higher than in sedentary western populations, although not exceedingly high. The aim of the present study was to analyze the relationships between dietary FA, as reflected by serum CE–FA composition, and serum lipids, lipoproteins, and apolipoproteins in this particular population.

MATERIALS AND METHODS

A description of the Kitava study population and its lifestyle, our methods of age estimation, sampling procedures, and the examinations of nonattendants have been given previously (18). Ethical approval was given by the national Medical Research Advisory Committee of Papua New Guinea. The survey was also approved by other national and provincial bodies and at the community level by the inhabitants and their chiefs. One of us (SL) spent seven weeks in late 1990 with a local family while eating the typical food and closely observing the dietary habits of the host family and of more than 30 other inhabitants throughout the island. A diet history was

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Abbreviations: ApoA1, serum apolipoprotein A1; ApoB, serum apolipoprotein B; BMI, body mass index; CE, serum cholesterol ester; en%, dietary energy; FA, fatty acid; HDL-C, serum high density lipoprotein-cholesterol; IHD, ischemic heart disease; LDL-C, serum low density lipoprotein-cholesterol; Lp(a), serum lipoprotein(a); MLR, multiple linear regressions; PL, serum phospholipid; PUFA, polyunsaturated fatty acid; std β, standardized regression coefficients; TC, serum total cholesterol; TG, serum triglycerides.

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taken from five families, combined with weighing of constituents of ready-to-eat portions among three families. Study participants were interviewed about the amounts of modern foodstuffs consumed in the last 12 mon. Macronutrient composition was calculated from international food tables.

Ages were estimated by the use of historic events as reference. Fasting blood samples were obtained in the morning after an overnight fast from 129 males and 51 females aged 13–86 yr, 67% of which were randomly selected. Electricity for the centrifuge was provided by a petrol-powered generating set. Samples were kept at –130°C in a liquid nitrogen vessel (Air Liquide GT35; Air Liquide, Champigny, France) until storage at –70°C in Sweden. Blood pressure was recorded with subjects in the seated position. Age estimates, smoking status (no/yes), anthropometry, serum lipids, and CE–FA were available for 151 subjects.

The CE–FA were determined by using gas–liquid chromatographic methods as described earlier (21), and TC, HDL-C, and TG by using enzymatic methods (22). LDL-C was estimated according to Friedewald. The concentrations of apolipoprotein B100 (ApoB) and ApoA1 were determined by immunoturbidimetry in the Multistat III F/LS apparatus using monospecific polyclonal antibodies. Before assay, the serum samples were preincubated with TG lipase (23). Lp(a) was measured by the Pharmacia Apo(a) RIA method. This is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the Apo(a) in the sample. The concentration is expressed in units/L (U/L). One U of Apo(a) is approximately equal to 0.7 mg of Lp(a) according to the manufacturer. Serum analyses for TG, TC, and HDL-C were performed in one series, and CE–FA, ApoB, ApoA1, and Lp(a) in another series, all within 14 mon after sampling.

Statistical analyses were made separately for males and females. Each continuous variable was checked for or transformed into apparent normality by repeated use of normal probability plots (24). The only variables which appeared normally distributed without transformation in both sexes were CE18:2n-6 and ApoA1, BMI and HDL in males, and CE14:0 in females. After transformations, nine outliers were excluded which were all 3–6 standard deviations from respective means. Age trends were reasonably linear as assessed from bivariate scatter plots and from residual plots in the regression models.

Forward stepwise multiple linear regressions (MLR) were performed using Statistica software for the Macintosh (25). Age, smoking status (no/yes), and CE-FA were included as independent variables. Standardized regression coefficients (std β) are given to show the relative impact, in the final step, of each independent variable. The value of std β demonstrates how many standard deviations the (transformed) dependent variable changes when the (transformed) explanatory variable changes one standard deviation, given that the other entered variables are kept constant. Cumulative adjusted R squared are given to show, at each step, the percentage of variation of the dependent variable so far explained by the model.

RESULTS

Clinical characteristics. Kitavans were very lean and their diastolic blood pressure was low, while the prevalence of smokers was remarkably high (Table 1). Serum TC and LDL-C in males were similar to contemporary Japanese levels, whereas in young and middle-aged females they were at least as high as in Sweden.

Dietary pattern. Yam, sweet potato, fruit, fish, and coconut were dietary staples. Most notable was the virtual absence of dairy products, butter, margarine, lard, oils, refined sugar, alcohol, and cereals, foods which provide more than 70% of the dietary energy (en%) in Sweden. Saturated fat intake from coconut was high (mainly lauric and myristic acid), as well as carbohydrate intake from tubers and fruit, while the intake of total, monounsaturated and polyunsaturated fat was low (Tables 2 and 3). Alcohol was not available.

FA composition of CE. As is evident from Table 4, most CE–FA differed substantially from typical western populations. Palmitic (16:0) and palmitoleic acid (16:1n-7) were high. Linoleic acid and its derivatives were very low, and all n-3 polyunsaturated fatty acids (PUFA) were high. Lauric acid (12:0) was only detected in trace amounts despite a very high intake.

Associations between lipids/lipoproteins/apolipoproteins and CE–FA. In both sexes the CE–FA composition explained much of the variation of TG, HDL-C and ApoA1, but less of the variation of TC, LDL-C, ApoB, and Lp(a) (Tables 3, 5, and 6). Smoking was associated with lower ApoA1 in both sexes independently of CE–FA, and in males with lower HDL-C and higher TG, TC, LDL-C, LDL/HDL, ApoB, and ApoB/A1. BMI was univariately and multivariately related to several CE–FA, but not to lipids, lipoproteins, or apolipoproteins after adjustment for age, smoking, and CE–FA.

TABLE 1
Clinical Characteristics of Kitavans[a]

	Males (n = 102)			Females (n = 40)		
	Mean	(SD)	Range	Mean	(SD)	Range
Age (yr)	49.3	(17)	20–81	52.9	(16)	20–83
Body mass index (kg/m^2)	20.3	(2.1)	15.1–25.9	18.2	(2.1)	14.5–22.6
Serum triglycerides (mmol/L)	1.14	(0.4)	0.5–3.0	1.20	(0.5)	0.6–2.7
Total cholesterol (mmol/L)	4.80	(0.8)	3.2–7.0	5.87	(1.2)	4.0–8.7
LDL-C (mmol/L)	3.23	(0.8)	1.6–5.6	4.05	(1.1)	2.2–6.5
HDL-C (mmol/L)	1.06	(0.2)	0.5–1.6	1.28	(0.3)	0.9–2.0
LDL-C/HDL-C	3.18	(1.1)	1.2–6.6	3.32	(1.2)	1.4–7.3
ApoB (g/L)	0.99	(0.2)	0.6–1.4	1.15	(0.2)	0.8–1.7
ApoA1 (g/L)	0.99	(0.2)	0.7–1.3	1.14	(0.2)	0.9–1.6
ApoB/apoA1	1.02	(0.2)	0.5–1.7	1.03	(0.2)	0.7–1.7
Lp(a) (mg/L)	165	(234)	8–1588	186	(241)	12–1154
Smoking rates (%)	81			81		

[a]LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; Apo, apolipoprotein; Lp(a), lipoprotein(a).

TABLE 2
Dietary Macronutrient Composition[a]

	Kitava	Sweden	RDA
Total fat	21	37	≤30
Saturated	17	16	<10
Monounsaturated	2	16	>10
Polyunsaturated	2	5	5–10
Protein	10	12	10–15
Carbohydrate	69	48	55–60
Alcohol	0	2	?

[a]Estimated dietary macronutrient composition expressed as percent of total energy in Kitava, among the general Swedish population, and as recommended to general western populations (RDA recommended dietary allowances).

TABLE 3
Estimated Dietary Intakes in Kitava[a]

Food	Weight (g)	Protein (g)	Fat (g)	Carbohydrate (g)	Energy (kJ)
Yam, sweet potato, taro	1200	25	2	300	5600
Fruit	400	3	<1	50	920
Coconut	110	4	43	7	1865
Fish	85	17	4	0	445
Other vegetables	200	5	<1	14	360
Western food	<1	0	<1	<1	20
Total	2000	54	50	370	9200

[a]Values are estimated daily medians for adults.

TABLE 4
Fatty Acid Composition of Serum Cholesterol Esters[a]

Fatty acid	Males	Females	Comment
Lauric 12:0	trace	trace	<–>
Myristic 14:0	3.9 (1.0)	4.1 (1.0)	Very high
Palmitic 16:0	14.7 (1.3)	14.3 (1.0)	Very high
Stearic 18:0	.79 (.2)	.77 (.2)	Low
Saturated FA	19.4 (1.6)	19.1 (1.5)	Very high
Palmitoleic 16:1n-7	6.8 (2.5)	8.7 (3.2)	Very high
Oleic 18:1n-9	17.0 (2.8)	18.2 (3.0)	<–>
Vaccenic 18:1n-7	1.7 (.4)	1.7 (.3)	High
Monounsaturated FA	25.1 (5.0)	28.5 (5.9)	High
Linoleic 18:2n-6	42.6 (5.5)	39.7 (5.4)	Very low
γ-Linolenic 18:3n-6	.48 (.2)	.55 (.3)	Low
Dihomo-γ-linolenic 20:3n-6	.53 (.1)	.55 (.1)	Low
Arachidonic 20:4n-6	6.42 (1.9)	6.30 (1.4)	<–>
α-Linolenic 18:3n-3	1.2 (.4)	1.5 (.4)	High
Eicosapentaenoic 20:5n-3	2.6 (1.4)	2.3 (.9)	High
Docosahexaenoic 22:6n-3	1.4 (.5)	1.5 (.5)	High
Polyunsaturated FA	55.1 (5.4)	52.3 (5.4)	Low
n–3/n–6	.11 (.04)	.11 (.03)	Very high

[a]Percent of total fatty acids (FA) of serum cholesterol esters, means (SD). Comments refer to comparisons with Western populations.

DISCUSSION

In these traditional Pacific Islanders, who apparently escape atherothrombotic cardiovascular disease despite high smoking rates and partly unfavorable serum lipid levels, and despite a high intake of saturated fat, 11–43% of the variation of serum lipids, lipoproteins, and apolipoproteins was explained by age, smoking, and FA composition of CE.

TABLE 5
Variables Explaining Variation in Serum Lipoproteins, Apolipoproteins, and Body Mass Index in Multivariate Analysis Among Kitavan Males[a]

Dependent variables	Explanatory variables	std β in final step	Cumulative adj. R^2, %
TG	16:1n-7	.26[b]	19
	18:1n-7	–.28[c]	25
	16:0	.50[d]	28
	20:5	–.54[d]	36
	Smoking	.23[c]	40
TC	Smoking	.18[b]	4
	16:0	–.52[d]	17
	18:2	–.47[d]	20
LDL-C	16:1n-7	.31[c]	7
	Smoking	.23[b]	12
HDL-C	16:0	–.72[d]	12
	20:5	.62[d]	30
	Smoking	–.29[d]	37
	18:3n-6	.16[b]	39
LDL/HDL-C	16:1n-7	.33[d]	9
	Smoking	.29[c]	16
ApoB	16:1n-7	.38[d]	13
	Smoking	.20[b]	16
ApoA1	16:0	–.55[d]	5
	20:5	.66[d]	13
	18:1n-9	.38[d]	20
	Smoking	–.24[c]	25
	18:1n-7	–.25[b]	29
ApoB/A1	Smoking	.34[d]	7
	18:1n-7	.28[c]	13
	20:5	–.49[d]	21
	16:0	.34[c]	27
Apo(a)	14:0	–.26[c]	6
	Age	.34[c]	9
	16:1n-7	–.24[b]	12
Body mass index	Age	–.46[d]	25
	16:0	.58[d]	36
	18:1n-7	–.29[c]	43
	18:3n-3	–.28[d]	48
	22:6n-3	–.28[b]	51

[a]Significant independent variables, standardized regression coefficients (std β), and adjusted R squared (adj. R^2) in forward stepwise multiple regressions including age, smoking status, and 13 cholesterol ester fatty acids as independent variables. Abbreviations as in Table 1; TG, triglycerides; TC, total cholesterol. [b]$P < .05$. [c]$P < .01$. [d]$P < .001$.

The estimated FA intake was, to a certain degree, reflected in the CE–FA composition, with two possible exceptions—CE12:0 was only detected in trace amounts, despite a very high intake from coconut, and CE16:0 was high despite a very low intake. The latter finding is probably explained by a high rate of endogenous fat synthesis at low total fat intake. The proportion of n-3 PUFA was high and that of n-6 PUFA was low in consistency with the high intake of coconut and fish and the absence of oils, margarine, dairy products, and meat. The high level of CE14:0 agrees with the high amount of 14:0 in coconut.

TABLE 6
Variables Explaining Variation in Serum Lipoproteins, Apolipoproteins, and Body Mass Index in Multivariate Analysis Among Kitavan Females[a]

Dependent variables	Explanatory variables	std β in final step	Cumulative adj. R^2, %
TG	20:5	$-.56^d$	22
	16:0	$.43^c$	31
	18:1n-7	$-.31^b$	38
TC	18:3n-3	$.36^b$	11
LDL-C	18:3n-3	$.38^b$	12
HDL-C	16:0	$-.60^d$	18
	20:5	$.40^b$	30
LDL/HDL-C	18:3n-3	$.55^d$	13
	16:1n-7	$-.39^b$	20
	20:3n-6	$.34^b$	29
ApoB	18:3n-3	$.63^d$	19
	16:1n-7	$-.43^c$	29
	20:3n-6	$.31^b$	37
ApoA1	16:0	$-.56^c$	22
	Smoking	$-.41^c$	32
	Age	$.33^b$	34
	18:0	$.36^b$	41
ApoB/A1	18:3n-3	$.57^d$	10
	20:3n-6	$.61^d$	20
	18:3n-6	$-.56^d$	43
Apo(a)	20:3n-6	$.44^c$	17
Body mass index	Age	$-.39^b$	13

[a]Significant independent variables, standardized regression coefficients, and adjusted R squared in forward stepwise multiple regressions including age, smoking status, and 13 cholesterol ester fatty acids as independent variables. Abbreviations as in Tables 1 and 4. $^bP < .05$. $^cP < .01$. $^dP < .001$.

Long-chain n-3 FA. In both sexes, CE20:5n-3 was negatively related to TG and positively related to HDL-C, and in males it was positively related to ApoA1. These relations are consistent with findings in a large number of dietary intervention trials and observational studies in western populations (26,27).

The second major FA reflecting fish intake, CE22:6n-3, behaved similarly to CE20:5n-3, although the association with lipoproteins and apolipoproteins reached significance only after excluding CE20:5n-3 as an independent variable (data not shown). When males and females were analyzed together in MLR, CE22:6n-3 was positively related to HDL-C even after adjustment for CE20:5n-3 (std $\beta = 0.32$; $P = 0.0036$). Recent reports have disputed this similarity between 20:5n-3 and 22:6n-3 in relation to serum lipids. In a coastal Norwegian population of males and females, HDL-C and ApoA1 were thus shown to be negatively related to 22:6n-3 in serum phospholipids (PL) and positively related to PL20:5n-3 (27). Furthermore, TG was not related to PL22:6n-3 in the same Norwegians, nor in French males (28). Contrasting findings suggest that fish oils rich in 22:6n-3 increase HDL-C to a greater degree than those rich in 20:5n-3 (29). A problem when interpreting cross-sectional findings is that 20:5n-3 and 22:6n-3 seem to differ in their time-course of incorporation into plasma lipids and in their affinity to lecithin:cholesterol acyl transferase, which is believed to form most of the CE present in plasma (30). Another problem is the possible confounding effect of alcohol intake, which was negatively associated with PL22:6n-3 and positively with PL20:5n-3 in French males (28), and which is known to raise HDL-C, ApoA1, and TG, and to lower LDL-C, at least in heavy drinkers (31). Alcohol intake was close to nil in Kitava.

Saturated fat. The negative association between CE16:0 and TC in males is at first sight surprising, as dietary 16:0 is positively related to LDL-C and TC, at least when the intakes of cholesterol and total fat are high or when the LDL receptor activity is otherwise compromised (32), and also because dietary 16:0 is positively related to CE16:0 at constant fat intake around 30 en% (10). However, when total fat intake is low, CE16:0 may preferentially reflect endogenous fat synthesis from carbohydrates rather than dietary intake of 16:0 (33). This may also explain the fact that CE16:0 in both sexes was positively related to TG and negatively to HDL-C, as TG is elevated, and HDL-C is reduced by an increased intake of carbohydrates (34), at least when dietary fat provides less than 25 en% and carbohydrates more than 60 en%, as is the case in Kitava. Similar negative relations between CE16:0 and HDL-C in healthy males from Finland (35) and France (36) suggest, on the other hand, that there may be additional explanations.

Myristic acid (14:0), which is the second most prevalent FA in coconut and probably the best marker of coconut intake in this survey, has repeatedly been shown to raise TC, LDL-C, and ApoB (37). Because the high intake of coconut is a probable cause of the fairly high TC, LDL-C, and ApoB in Kitavans, the absent relationship between CE14:0 and these variables is unexpected.

A diet high in 12:0 and 14:0 given to Danish men resulted in lower levels of Lp(a) (38), which may be beneficial in terms of IHD. It is therefore interesting that in Kitavans a univariate inverse relation was found between Lp(a) and CE14:0 in males. Nevertheless, despite a very high intake of 12:0 and 14:0 (and a negligible intake of *trans* FA), levels of Lp(a) were essentially the same as in Sweden (19), which is expected as much evidence has shown that Lp(a) is for the most part genetically determined (39).

Monounsaturated fat. In males CE16:1n-7 was positively related to TG, LDL-C, and ApoB. Similar relations were found in a large sample of middle-aged French males where, furthermore, CE16:1n-7 was positively associated with HDL-C and ApoA1 (36). These relationships were only significant in drinkers, and it was concluded that alcohol consumption was the probable cause, which agreed with other studies showing an association between alcohol consumption and 16:1n-7 in CE or PL in males (8,28,40). Alcohol intake is

negligible in Kitava, as already stated. Most of the 16:1n-7 appearing in CE in Kitavans is expected to be derived from 16:0, which would explain most of the behavior of CE16:1n-7 in multivariate analysis among males. However, CE16:1n-7 was not univariately related to CE16:0 in males or females. Rather, CE16:1n-7 was strongly and negatively related to CE18:2n-6, which explained 43 and 45% of the variation of CE16:1n-7 in stepwise MLR among males and females, respectively (data not shown), and this is partly in accordance with earlier findings (8,10).

n-6 FA. As expected, CE18:2n-6 was strongly and negatively associated with TC, but only in males. This agrees with consistent evidence that linoleic acid reduces LDL-C (41). The lack of relation to HDL-C and ApoA1 supports the notion that dietary 18:2n-6 has little effect on these two variables at realistic intakes (<10 en% in the West; <5 en% in Kitava) (42).

In females, CE20:3n-6 was positively associated with LDL/HDL, ApoB, and Lp(a), which is consistent with the higher levels of CE20:3n-6 in patients with IHD (21) or impaired glucose tolerance (43). Among French males, CE20:3n-6 was positively related to TG, TC, LDL-C, LDL/HDL, ApoB, and ApoB/A1, and negatively to HDL-C and ApoA1 (36). From dietary intervention trials, there is evidence of a strong inverse relationship between the intake of 18:2n-6 and its metabolite 20:3n-6 after two weeks (42), as well as after five months (10).

BMI. Contrary to what has been found in western populations, BMI was unrelated to lipoproteins and apolipoproteins after adjustment for age, smoking, and CE–FA, and the weak relations that tended to exist were opposite those generally expected in westerners (data not shown). This is not surprising as BMI in this very lean population probably reflects muscle mass rather than body fat.

Conclusions. In this population of subsistence horticulturalists, several relations were found between serum CE and serum lipoproteins and apolipoproteins, and it is inferred that this reflects the effects of dietary fatty acids. In earlier cross-sectional studies, mainly in westerners, significant correlations between the FA of human tissues and serum lipoproteins or apolipoproteins have often been weak and inconsistent, though less so for 18:2n-6 (9,13,16,28,32,36,44–50). The reasons for the unexpectedly large correlations in the present study are not evident. Partial explanations may be that the population is homogeneous and stable, and that important confounding factors of nutrition appear to be absent.

The findings also confirm that smoking is associated with raised TG, TC, LDL-C, and ApoB and with lowered HDL-C and ApoA1 (51), and that these relationships are largely independent of diet and alcohol.

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Stearic Acid Unlike Shorter-Chain Saturated Fatty Acids Is Poorly Utilized for Triacylglycerol Synthesis and β-Oxidation in Cultured Rat Hepatocytes

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ABSTRACT: Utilization of stearate as compared to various saturated fatty acids for cholesterol and lipid synthesis and β-oxidation was determined in primary culture of rat hepatocytes. At 0.5 mmol/L in the medium, stearate (18:0) adequately solubilized by albumin was less inhibitory to cholesterol synthesis from [2-^{14}C] acetate than myristate (14:0) and palmitate (16:0) (68% vs. 91 and 88% inhibition, respectively). The rate of incorporation into cholesterol from [1-^{14}C] stearate (3.0 ± 0.6 nmol/mg protein/4 h) was 37-, 1.8-, and 7.8-fold of that from myristate, palmitate, and oleate, respectively. Conversely, the rate of [1-^{14}C] stearate incorporation into total glycerolipids was 88–90% lower than that of labeled palmitate, myristate, and oleate. The rate of [1-^{14}C] stearate incorporation into triacylglycerol (3.6 ± 0.4 nmol/mg protein /4 h) was 6–8% of that from myristate, palmitate, oleate, and linoleate. The rate of stearate incorporation into phospholipids was the lowest among tested fatty acids, whereas the rate of mono-and diacylglycerol synthesis was the highest with stearate treatment. The rate of β-oxidation as measured by CO_2 and acid soluble metabolite production was also the lowest with [1-^{14}C] stearate treatment at 22.7 nmol/mg protein/4 h, which was 35–40% of those from other [1-^{14}C] labeled fatty acids. A greater proportion of stearate than other fatty acids taken up by the hepatocytes remained free and was not metabolized. Clearly, stearate as compared to shorter-chain saturated fatty acids was less efficiently oxidized and esterified to triacylglycerol in cultured rat hepatocytes.
Lipids 31, 159–164 (1996).

Even though the mortality from coronary heart disease has significantly decreased in the last two decades, the disease remains a major health problem and the leading cause of death in the United States (1,2). Coronary heart disease is a multifactorial disease, and high level of plasma cholesterol, especially low density lipoprotein (LDL) cholesterol, is one of the major risk factors that can be modified (2,3). Results from many animal, epidemiological, and clinical studies suggest that the amount and type of dietary fat affect plasma cholesterol concentrations (4–7). Unlike other shorter-chain saturated fatty acids [i.e., laurate (12:0), myristate (14:0), palmitate (16:0)] which are hypercholesterolemic, stearate (18:0) does not raise plasma cholesterol concentrations (5,8–14). Poor digestibility and bioavailability (15–18), absence of LDL receptor downregulation (19), and conversion of stearate to oleate (20,21) have been suggested as possible mechanisms underlying the neutral effect of stearate on plasma cholesterol. However, these mechanisms together do not fully explain the extent of the hypocholesterolemic nature of stearate. It is reasonable to assume that other mechanism(s) may also be involved in the regulation by stearate.

The poor understanding of cellular mechanism underlying the effect of dietary saturated fatty acids on plasma cholesterol concentration is attributed to its complexity of interaction among different fatty acids. Therefore, it is important to understand the mechanism regulating metabolism of individual fatty acids. By using primary culture of rat hepatocytes, the specific effects of fatty acids on lipid metabolism can be determined without the complications of others. The present study was conducted to determine the effects of different saturated fatty acids on cholesterol synthesis from acetate, and to determine utilization of different saturated fatty acids for cholesterol and glycerolipid synthesis, and for oxidation. The results showed that despite its high cholesterogenesity, the utilization of stearate for either oxidation or synthesis of glycerolipids, especially triacylglycerol, was markedly lower than other fatty acids.

MATERIALS AND METHODS

Animals and diets. Sprague-Dawley male rats (100–150 g body weight) were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and fed *ad libitum* a rat chow from Ralston Purina Co. (St. Louis, MO). The rats were housed individually in stainless-steel cages at approximately 24°C and 50% relative humidity on a 12-h light/dark cycle (0600–1800) and had free access to water at all times. They were sacrificed between 0900 and 1000 on the day of hepatocyte isolation. The animal protocol was approved by The Pennsylvania State University Institutional Animal Care and Use Committee.

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Abbreviations: ASM, acid-soluble metabolites; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; LDL, low density lipoprotein.

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Preparation of rat hepatocytes. Hepatocytes were isolated from rats (225–250 g body weight) by the method of Berry and Friend (22) as modified by Seglen (23). As detailed elsewhere (24), the procedure involved collagenase perfusion *via* the hepatic portal vein, tissue mincing, filtration and centrifugation, and further purification by cell adherence. The isolated cells were resuspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 5.6 mmol/L glucose, 10% fetal bovine serum (FBS), and antibiotics (penicillin, 100 units/mL and streptomycin, 100 μg/mL). The cell viability was determined by Trypan blue exclusion for each isolation. The cell preparations with 95% or higher viability were used throughout the experiments. A 2-mL aliquot of cell suspension ($0.7–0.8 \times 10^6$ cells/mL) was transferred to culture plates, each containing six wells (3.5 cm diameter), and incubated at 37°C under an atmosphere of 95% air and 5% CO_2. Six hours after incubation, cells that adhered to the flask were refed with the DMEM and incubated under the same conditions for 24 h. The adhered cells derived from the same liver were used for various fatty acid treatments in each experiment.

Hepatic capacity for cholesterol and lipid synthesis. The cultured cells obtained after 24 h incubation were washed three times with 2 mL of FBS-free DMEM. They were then incubated in the same medium with [2-^{14}C] acetate (specific activity, 36.96 MBq/mmol) and unlabeled acetate (1.0 mmol/L) in the presence of different fatty acids at 0.5 mmol/L. Fatty acids were provided as an albumin complex in a molar ratio of 5.6 (fatty acid/albumin). Stock solutions (100 mmol/L) were prepared from the sodium salt of each fatty acid. An aliquot of the stock solution was added to the DMEM containing 0.59% bovine serum albumin (BSA) followed by sonication prior to the incubation. At the end of 4 h incubation, the cells were harvested and lipids were extracted from the cells according to the procedure of Folch *et al.* (25). The extracted lipids were saponified and cholesterol was precipitated with digitonin solution (26). Fatty acids were extracted from the saponifiable fraction. Radioactivity of cholesterol and fatty acids derived from [2-^{14}C] acetate was measured using a liquid scintillation spectrometer (LS8100; Beckman instruments, Inc., Palo Alto, CA). The rate of cholesterol synthesis was expressed as nmol of acetate incorporated/mg protein/4 h. Cellular protein was determined by the method of Lowry *et al.* (27) from hepatocytes harvested from the culture treated without fatty acid and BSA during the 4-hr incubation.

Cholesterogenesity and lipogenesity of fatty acids. To evaluate the effects of individual fatty acids on the cholesterol and glycerolipid synthesis, the cultured cells obtained after 24 h incubation were washed three times with 2 mL of FBS-free DMEM followed by incubation in the same medium containing 0.5 mmol/L [9,10-^{3}H]myristate, [1-^{14}C]palmitate, [1-^{14}C]stearate or [1-^{14}C] oleate and corresponding nonlabeled fatty acid to yield a specific activity of 37 MBq/mmol. After 4 h incubation, the cellular lipids were extracted. For analysis of glycerolipids synthesized from the labeled fatty acids, the extracted cellular lipid was separated into phospholipid, triacylglycerol, diacylglycerol, monoacylglycerol, and free fatty acids on Silica Gel H plates using a solvent system consisting of hexane/ethyl ether/acetic acid (80:20:1) (28). Each lipid class was reextracted prior to radioactivity determination by liquid scintillation counting. The rate of synthesis was expressed as nmol of substrate incorporated/mg protein/4 h.

β-Oxidation of fatty acids. For measurement of β-oxidation capacity, the incorporation of [1-^{14}C] labeled fatty acid into $^{14}CO_2$ and acid soluble metabolites (ASM) was determined (29). ASM measured under the conditions were predominantly ketone bodies with Krebs'cycle intermediates and acetyl CoA as minor components (30). Cell treatments were identical to those in the cholesterol and lipid synthesis experiments described above except that culture flasks (25 cm^2) instead of the culture plates were used. Each culture flask was sealed with a rubber cap containing a center well. After 4 h incubation, 0.2 mL of 35% perchloric acid was injected to the medium to stop the reaction. To the filter paper in the center well, 0.1 mL of 1 M hyamine hydroxide solution was injected (30). The culture flasks were shaken for 1 h on ice to trap CO_2. For CO_2 measurement, the center well was placed in a scintillation vial and dried overnight. The radioactivity of CO_2 was determined by liquid scintillation counting. For measurement of ASM, the culture medium was centrifuged, and the supernatant was used for radioactivity determination. The rate of product formation was expressed as nmol of substrate incorporated/mg protein/4 h.

Fatty acid binding to albumin. The binding of fatty acids to BSA was determined by measuring the partitioning of [^{14}C] labeled fatty acids between heptane and DMEM (31,32). The binding of fatty acid at various fatty acid to BSA molar ratios (2:1, 5.6:1, 10:1) was tested. Fatty acid was maintained at 0.5 mmol/L in the medium. An aliquot of the stock solution of each fatty acid (100 mmol/L) was added to 20 mL of DMEM containing 1.65, 0.59, 0.33% BSA and [1-^{14}C]palmitate, [1-^{14}C]stearate, or [1-^{14}C]oleate with a specific activity of 3.7 MBq/mmol followed by sonication prior to the incubation. The mixture was then transferred to a 50-mL capped tube. A 2-mL portion of heptane was added, and the tubes were shaken at 20 strokes per minutes for 48 h to achieve equilibrium. After equilibration, the heptane fraction was transferred to scintillation vials and evaporated. The radioactivity was measured in a liquid scintillation spectrometer. The results expressed as percentage binding were calculated by subtracting the radioactivity in the heptane fraction from total radioactivity in the medium, dividing the value by total radioactivity in the medium and multiplying by 100.

Materials. Collagenase D was purchased from Boehringer Mannheim Co. (Indianapolis, IN). [2-^{14}C] Acetate, [9,10-^{3}H] myristate, [1-^{14}C] palmitate, [1-^{14}C] stearate, [1-^{14}C] oleate, and [1-^{14}C] linoleate were products of Amersham Co. (Arlington Heights, IL). Fatty acid-free BSA and sodium salts of myristate, palmitate, stearate and oleate were purchased from Sigma Chemical Co. (St. Louis, MO). DMEM, FBS, and antibiotics (penicillin and streptomycin) were provided by Life

Technologies, Inc. (Grand Island, NY). Silica gel H plates (20 × 20 cm, 250 μm layer thickness) were obtained from Alltech Associates, Inc. (Deerfield, IL).

Statistical analysis. Data were expressed as mean ± SD. The significant differences among various fatty acid treatments were analyzed by analysis of variance. Duncan's multiple range test was applied to identify significant differences between treatment group means (33). Probability values less than 0.05 were judged to be statistically significant.

RESULTS

Cholesterol synthesis. The rate of [2-^{14}C] acetate incorporation into cholesterol and fatty acid was measured in the presence or absence of 0.5 mmol/L myristate, palmitate, stearate, or oleate. All fatty acids tested were found to be inhibitory to cholesterol synthesis from [2-^{14}C] acetate when compared with the rate obtained from the cells incubated without exogenous fatty acid. Oleate and myristate were the most inhibitory (92 and 91%, respectively), followed by palmitate (88%) and stearate (68%) (Fig. 1). Clearly, stearate is less potent than shorter-chain saturated fatty acids and oleate in inhibiting cholesterol synthesis. Stearate was as effective as oleate and palmitate in inhibiting [2-^{14}C] acetate incorporation into fatty acid by 92–95%. Myristate inhibited the fatty acid synthesis by 73%. The experiments were then conducted to determine the rate of [9,10-^{3}H] myristate, [1-^{14}C] palmitate, [1-^{14}C] stearate, and [1-^{14}C] oleate incorporation into cholesterol. The rate of substrate incorporation into cholesterol, expressed as nmol/mg protein/4 h, were in decreasing order: stearate (2.98 ± 0.52) > palmitate (1.69 ± 0.24) > oleate (0.38 ± 0.14) > myristate (0.08 ± 0.008).

Glycerolipid synthesis. In view of the potential competition of cholesterol and glycerol lipid synthesis for the fatty acid substrate, the rates of fatty acid utilization for glycerol lipid production were determined. The rate of stearate incorporation into phospholipids was the lowest (2.9 ± 0.3 nmol/mg prot/4 h) among the tested fatty acids (Fig. 2). On the other hand, the rate of monoacylglycerol and diacylglycerol synthesis was the highest with stearate treatment. More strikingly, the rate of stearate incorporation into triacylglycerol (3.6 ± 0.4 nmol/mg protein/4 h) was only 6.3, 6.1, 5.6, and 7.5% of that of oleate, myristate, palmitate, and linoleate, respectively. Consequently, the highest rate of incorporation into glycerolipids (a sum of phospholipids, tri-, di- and monoacylglycerols) was noted for palmitate (72.4 ± 5.6 nmol/mg protein/4 h), followed by myristate (64.4 ± 3.0), oleate (62.7 ± 4.1), linoleate (55.1 ± 2.0), and stearate (6.5 ± 0.4 nmol/mg/4 h).

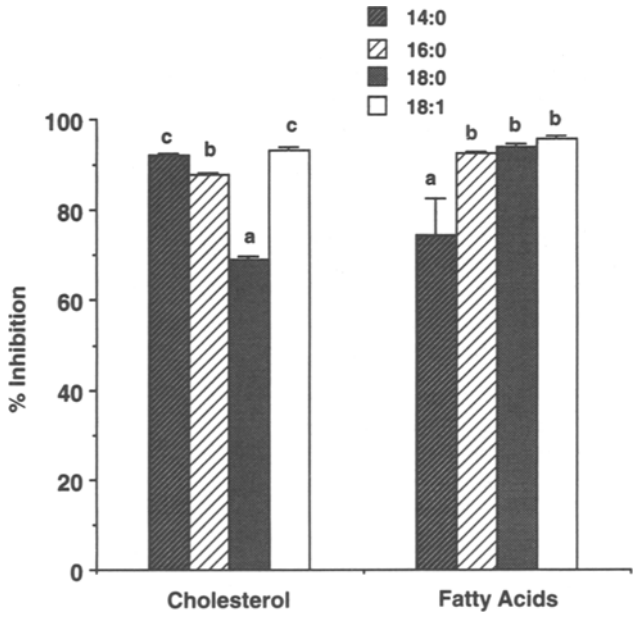


FIG. 1. Inhibition of [2-^{14}C] acetate incorporation into cholesterol and fatty acid by exogenous fatty acids in cultured rat hepatocytes. Values are mean ± SD for four samples. Values without a common letter are significantly different at $P < 0.05$. Percent inhibition is defined as the difference between the amount of [^{14}C]acetate recovered in cholesterol or fatty acids in the fatty acid treated hepatocytes and that in the hepatocytes without exogenous fatty acids divided by the amount of [^{14}C] acetate recovered in cholesterol or fatty acids in the hepatocytes without any fatty acid treatment multiplying by 100. The rate of acetate incorporation into cholesterol and fatty acid was 1.05 ± 0.03 and 0.26 ± 0.01 nmol/mg protein/4 h, respectively, for the control group. For the bars with unnoticeable error bars, the error bar is thinner than the bar frame. Myristate, palmitate, stearate, and oleate treatments are represented by 14:0, 16:0, 18:0, and 18:1, respectively.

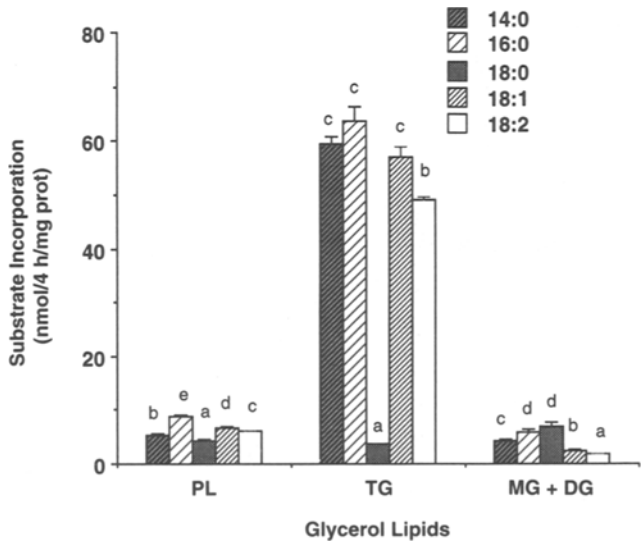


FIG. 2. Incorporation of [9,10-^{3}H] or [1-^{14}C] labeled fatty acids into various glycerolipids in cultured rat hepatocytes. Values are mean ± SD for four samples. Values without a common letter in the same cluster are significantly different at $P < 0.05$. Substrate incorporation is defined as nmol fatty acid incorporated into various glycerolipids for four hours per mg cellular protein. For the bars with unnoticeable error bars, the error bar is thinner than the bar frame. Myristate, palmitate, stearate, oleate, and linoleate treatments are represented by 14:0, 16:0, 18:0, 18:1, and 18:2, respectively. Abbreviations: PL, phospholipids; TG, triacylglycerols; MG + DG, monoacylglycerol and diacylglycerol.

TABLE 1
Utilization of [1-^{14}C] Labeled Fatty Acids *via* Mitochondrial β-Oxidation in Cultured Rat Hepatocytes[a]

Product	[^{14}C] Labeled			
	Palmitate	Stearate	Oleate	Linoleate
	(nmol/mg protein/4 h)			
$^{14}CO_2$	9.6 ± 4.1[d]	2.9 ± 0.47[c]	13.2 ± 2.1[d]	13.1 ± 1.2[d]
ASM	54.2 ± 12.9[d]	19.8 ± 3.37[c]	78.8 ± 15.2[e]	75.2 ± 11.4[e]

[a]Values are means ± SD for six to eight samples. Values without a common superscript in the same row are significantly different at $P < 0.05$. ASM, acid-soluble metabolites containing predominantly ketone bodies (Ref. 30).

Oxidation of fatty acids. The incorporation of [1-^{14}C] labeled fatty acid into CO_2 and ASM (29) was taken as a measure of β-oxidation capacity. The rates of CO_2 and ASM formation from [1-^{14}C] stearate were lowered than those from other [1-^{14}C] labeled fatty acids (Table 1). Thus, the sum of these two products derived from [1-^{14}C] stearate (22.7 nmol/mg protein/4 h) was 60–75% of that from other [1-^{14}C] labeled fatty acid.

The analysis of radiolabeled fatty acids accumulated in hepatocytes revealed that the amount of free fatty acid recovered in the cells was the highest with [1-^{14}C]stearate, followed by [1-^{14}C]palmitate, [1-^{14}C]oleate, [9,10-^{3}H]myristate, and [1-^{14}C]linoleate (Table 2). Specifically, the amount of the recovery of stearate, palmitate, oleate, myristate, and linoleate represented 8.5 ± 0.8%, 4.7 ± 0.8%, 0.7 ± 0.2%, 0.3 ± 0.001%, and 0.1 ± 0% of initial dose of the corresponding fatty acids (i.e., 1000 nmoles per incubation) in the incubation medium.

The sum of products (i.e., cholesterol, glycerolipids, β-oxidation metabolites, and free fatty acid) derived from [^{14}C] labeled fatty acids was then calculated. Of the initial dose of [1-^{14}C] fatty acids (1000 nmoles per incubation) provided in the medium, 12.5% (i.e., 124.7 ± 2.8 nmole/mg protein/4 h) of stearate as compared to 18.8% (i.e., 188.3 ± 5.0 nmol/mg protein/4 h) of palmitate and 16.3% (i.e., 162.5 ± 1.9 nmol/mg protein/4 h) of oleate were incorporated into all products.

Fatty acid binding. The different effects of fatty acids on lipid synthesis and β-oxidation led us to measure albumin binding capacity of fatty acids. The results showed that 99.84 ± 0.003% of stearate as compared to 99.45 ± 0.11% of palmitate and 99.94 ± 0.02% oleate was bound to BSA in the medium containing 2:1 molar ratio of fatty acid to BSA. Increasing the ratio to 5.6:1 and 10:1 did not alter the percentage of binding for stearate or palmitate, but slightly and significantly decreased oleate binding to 99.53 ± 0.22% and 96.54 ± 0.24%, respectively.

TABLE 2
Accumulation of [1-^{14}C] or [9,10-^{3}H] Labeled Free Fatty Acids in Cultured Rats Hepatocytes[a]

Substrate	Fatty acid accumulated (nmol/mg prot/4 h)
[9,10-^{3}H] Myristate	2.63 ± 0.01[c]
[1-^{14}C] Palmitate	47.04 ± 7.92[e]
[1-^{14}C] Stearate	84.60 ± 8.20[f]
[1-^{14}C] Oleate	6.75 ± 1.62[d]
[1-^{14}C] Linoleate	1.45 ± 0.00[b]

[a]Values are mean ± SD for six samples. Values without a common superscript in the same column are significantly different at $P < 0.05$. Free fatty acids were quantified by measuring cellular free fatty acids after 4 h incubation with [^{3}H] or [^{14}C] labeled fatty acids at 0.5 mM unlabeled fatty acid concentration.

DISCUSSION

Dietary fatty acids serve as precursors for cholesterol synthesis by producing acetyl-CoA *via* β-oxidation (34). They can also inhibit the cholesterol synthesis from other substrates such as acetate by diluting the acetyl-CoA pool. The rat liver plays important roles in controlling the plasma cholesterol concentration, because hepatic synthesis accounts for about 50% of whole animal cholesterol production (35). This together with the finding that stearate is as effective as oleate in reducing plasma cholesterol level (12) led us to speculate that stearate may impair hepatic cholesterol synthesis. Contrary to this speculation, we found that not only was stearate less inhibitory to cholesterol synthesis from acetate, but served as a preferred substrate over palmitate, myristate, and oleate for cholesterol synthesis. The most striking finding of the present study, however, is that the rate of stearate incorporation into triacylglycerol was markedly depressed to approximately 6–8% of that noted for myristate, palmitate, and oleate, suggesting a poor utilization of stearate for triacylglycerol synthesis (Fig. 2). The different rates of triacylglycerol synthesis from fatty acids have also been demonstrated previously by Nestel and Steinberg (36) and Kohour *et al.* (37). Consistent with the present study of hepatocytes, palmitate was incorporated into triacylglycerol at a rate that was 15–36% higher than that of linoleate incorporation in rat liver slices (36). Similarly, the output of triacylglycerol by the rat liver perfused with palmitate was the same as or higher than that perfused with oleate and linoleate (37). Interestingly, the rate of triacylglycerol output from stearate was not different or even higher than that from palmitate, depending on duration of the liver perfusion (37). The reason for the discrepancy in triacylglycerol synthesis from stearate observed between the cultured hepatocytes and perfused liver systems is not known. However, it is important to stress that maximal rate of triacylglycerol output by the liver perfused with stearate was accompanied by 480% increase in hepatic content of the fatty acid as compared to 46% increase of hepatocyte stearate in the present study (data not shown).

The mechanism underlying the marked impairment of triacylglycerol synthesis from stearate in cultured hepatocytes remains uncertain. Fatty acids present in the liver are either esterified to form glycerolipids or oxidized *via* β-oxidation (38,39). An alteration in one pathway can therefore inversely affect the other in metabolizing fatty acid. In fact, the inhibition of triacylglycerol synthesis by fish oil rich in n-3 fatty acids in rat liver and cultured hepatocytes (40,41) has been

attributed to increased CO_2 and ASM production, resulting in part from increased carnitine acyltransferase activity (41). Unlike the effect of n-3 fatty acid, the depressed rate of stearate incorporation into triacylglycerol could not be explained by enhanced ß-oxidation because the rates of CO_2 and ASM formation from [1-^{14}C] stearate were lower than those from other [1-^{14}C] labeled fatty acids (Table 1).

Triacylglycerol and phospholipid synthesis share common pathways and compete for fatty acids (42). Nonetheless, the impaired triacyglycerol synthesis from stearate was not a result of such a competition, since the rate of stearate incorporation into phospholipid was also decreased, although to a lesser extent. Dietary fatty acids are known to alter the activity of the enzymes involved in triacylglycerol synthesis: glycerol-3-phosphate acyltransferase, 1-acylglycerolphosphate acyltransferase, phosphatidate phosphohydrolase and 1,2-diacylglycerol acyltransferase (43). Our results showed that the rate of stearate incorporation into monoacylglycerol and diacylglycerol was similar to or higher than that of other fatty acids, suggesting that the activity of glycerol-3-phosphate acyltransferase and monoacylglycerol acyltransferase were not altered. It has been demonstrated in a feeding study that a markedly reduced plasma triacylglycerol concentration by fish oil was accompanied by approximately a 50% reduction in phosphatidate phosphohydrolase; however, the activity of diacylglycerol acyltransferase was not affected (43,44). Whether stearate inhibits phosphatidate phosphohydrolase and 1,2 diacylglycerol acyltransferase activity remains unclear.

In an attempt to further understand the possible mechanism, we found that the amount of exogenous stearate recovered as glycerolipids, cholesterol, β-oxidation products and free fatty acid in the hepatocytes was 25 and 35% lower than that derived from exogenous oleate and palmitate, respectively. Although β-oxidation was not determined in the experiment using [9,10- ^{3}H] myristate, the rate of myristate incorporation into the sum of glycerolipids and cholesterol (i.e., 64.5 nmol of myristate incorporated/mg protein/4 h) was 5.8-fold higher than that derived from stearate, indicating that similar to palmitate and oleate, myristate is more rapidly metabolized than stearate. It should be pointed out that the rate of myristate incorporation into the products may be underestimated due to potential exchange of [^{3}H] label of the fatty acid with water in the medium and cells. Taken together, the results of estimated recovery of cellular metabolites suggest that stearate uptake by the cells may be impaired. Although the reason for this impairment is obscure, the comparable albumin binding capacity of various fatty acids observed in the present study indicates that stearate was well solubilized in the medium. Nevertheless, the low substrate uptake alone could not fully account for greater than 90% reduction in triacylglycerol synthesis from stearate. It is intriguing that contrary to the low recovery of stearate in cellular metabolites, the amount of stearate remained free was the highest among all fatty acids. Of the total stearate taken up by the cells, more than 60% was free and not metabolized. Since activation of fatty acid is an essential step in its metabolism (45,46), the conversion of stearate to stearoyl CoA by fatty acyl CoA synthetase may be a limiting factor contributing to the impairment in stearate incorporation into triacylglycerol. This speculation is consistent with an earlier observation showing that the levels of fatty acyl CoA such as oleoyl CoA and linoleoyl CoA but not stearoyl CoA in liver and skeletal muscle reflected dietary fatty acids (47).

In summary, stearate is more cholesterogenic and less inhibitory to cholesterol biosynthesis from acetate than shorter-chain saturated and monounsaturated fatty acids. Most importantly, stearate is poorly utilized for triacylglycerol synthesis in cultured rat hepatocytes.

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Identification of Novel *trans* Isomers of 20:5n-3 in Liver Lipids of Rats Fed a Heated Oil

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ABSTRACT: *Trans* polyunsaturated n-3 fatty acids are formed as a result of the heat treatment of vegetable oils. It was demonstrated previously that the 18:3 Δ9*cis*, 12*cis*, 15*trans* containing a *cis* Δ9 ethylenic bond was converted to a geometrical isomer of 20:5n-3, the 20:5 Δ5*cis*, 8*cis*, 11*cis*, 14*cis*, 17*trans*. In the present study, we have identified two new isomers of eicosapentaenoic acid, the Δ11 mono*trans* and the Δ11,17 di*trans* isomers in liver of rats fed a heated oil. These are formed as a result of the conversion of two of the main isomers of linolenic acid which are present in refined and frying oils, the 18:3 Δ9*trans*, 12*cis*, 15*cis* and the 18:3 Δ9*trans*, 12*cis*, 15*trans*.

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Trans C_{18} polyunsaturated fatty acids (PUFA) are formed during heat treatments (deodorization, frying) of vegetable oils (1–3). Linoleic acid is mainly isomerized to 18:2 Δ9*cis*, 12*trans*, and 18:2 Δ9*trans*, 12*cis*, whereas α-linolenic acid is isomerized to three major isomers which are 18:3 Δ9*cis*, 12*cis*, 15*trans*, 18:3 Δ9*trans*, 12*cis*, 15*cis* and 18:3 Δ9*trans*, 12*cis*, 15*trans*. The double bond at the Δ12 position is only isomerized to a low degree (4). These *trans* C_{18} PUFA are present in oils and fats, but also in different food products (5,6). Consequently, they are present in human tissues, including serum (7) and milk (8,9). It is now established that the *trans* isomers of linoleic and α-linolenic acids which contain a *cis* double bond at the Δ9 position are converted into isomers of arachidonic acid (10,11), eicosapentaenoic acid (EPA), and docosahexaenoic acid (12). The latter have been reported in human platelets (13), suggesting that humans are able to desaturate and elongate *trans* C_{18} precursors. Some physiological effects of 20- and 22-carbon *trans* n-3 PUFA have been reported on platelet function and metabolism (14,15) and electroretinography (16).

Only a few studies have been performed on the metabolism of fatty acids containing a *trans* double bond at the Δ9 position. The conversion of 18:2 Δ9*trans*, 12*cis* was reported in mice (11), indicating that when this fatty acid contains a *trans* double bond at the Δ9 position, it also may be desaturated and elongated into an isomer of arachidonic acid (11,17). Moreover, 18:2 Δ9*trans*, 12*cis* might also be elongated (11). Elaidic acid (18:1 Δ9 *trans*) has also been shown to be Δ5 desaturated (18). However, the possible conversion of the *trans* isomers of linolenic acid containing a *trans* double bond at the Δ9 position (i.e., 18:3 Δ9*trans*, 12*cis*, 15*cis*, and Δ9*trans*, 12*cis*, 15*trans*), which are present in refined oil, had not yet been studied. Indeed, four unknown peaks were reported after gas–liquid chromatography (GLC) of a 20:5 fatty acid methyl esters (FAME) fraction isolated from liver lipids of rats fed a heated linseed oil (19). These peaks labelled as X1, X2, X3, and X4 in the original publication were suspected to be other 20:5 *trans* isomers, but they were not clearly identified. Two geometrical isomers of EPA (i.e., 20:5 Δ5*cis*, 8*cis*, 11*trans*, 14*cis*, 17*cis*, and 20:5 Δ5*cis*, 8*cis*, 11*trans*, 14*cis*, 17 *trans*), which were recently synthetized (20), enable us to identify two new 20:5 isomers as being the metabolites of 18:3 Δ9*trans*, 12*cis*, 15*cis*, and 18:3 Δ9*trans*, 12*cis*, 15*trans*.

MATERIALS AND METHODS

Liver lipids were extracted (21) from Wistar rats fed for six weeks a diet containing 10% of linseed oil heated at 275°C for 12 h under nitrogen, as previously described (12). This represented a daily intake of about 2.3 g of oil. FAME were prepared using boron trifluoride in methanol (14% by vol), according to Morrison and Smith (22). Then, FAME were fractionated by reversed-phase high-performance liquid chromatography, as previously reported (9). Briefly, a Nucleosil (particle size: 5 μm) C_{18} column (25 cm × 4.6 mm i.d., Shandon, Eragny, France) was used and the mobile phase was a mixture of acetonitrile and acetone (9:1 by vol) at a flow rate of 4 mL/min^{-1}. The fraction corresponding to the 20:5 FAME was collected and then analyzed by GLC using two columns of different polarities. The first one was a 30 m × 0.25 mm i.d. DB Wax column (film thickness: 0.25 μm; J&W Scientific, Rancho Cordo, CA) installed on a Packard 438 gas chromatograph (Packard Instruments, Les Ulis, France). The oven temperature was 190°C and the injector and flame-ionization detector were maintained at 230 and 260°C, respectively. The

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Abbreviations: DMOX, dimethyl oxazoline derivatives; ECL, equivalent chain lengh; EPA, eicosapentaenoic acid; FAME, fatty acid methyl esters; FTIR, Fourier transform infrared; GLC, gas–liquid chromatography; PUFA, polyunsaturated fatty acids.

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second column was a 50 m × 0.33 mm i.d. BPX 70 (film thickness: 0.25 μm; SGE, Melbourne, Australia) installed on a Hewlett-Packard 5890 series II gas chromatograph (Hewlett Packard, Les Ulis, France). The oven temperature was maintained at 170°C. The injector and detector were maintained at 250°C. Quantitative analyses were performed using a Chromjet SP 4490 integrator and Winer on Windows software (Thermo Separation Products, Les Ulis, France).

Additionally, the FAME were analyzed by gas-chromatographic–Fourier transform infrared (FTIR) using a CP Wax 57 CB column (50 m × 0.5 mm i.d.; Chrompack, Middelburg, The Netherlands) and a Bruker IFS 85 FTIR spectrometer (Bruker, Wissembourg, France) installed on a Carlo Erba (Milano, Italy) 5160 chromatograph. The conditions were the same as already described (12). Aliquots of the FAME were transformed into dimethyl oxazoline derivatives (DMOX) according to Fay and Richli (23). They were then analyzed by gas chromatography/mass spectrometry using a BPX 70 column as described previously and a Hewlett-Packard 5970 mass spectrometer. The temperature of the gas chromatographic oven was programmed from 60 to 170 at a rate of 20°C/min^{-1}.

RESULTS AND DISCUSSION

The FAME analysis of the 20:5 fraction from liver lipids of rats fed heated linseed oil showed six peaks as described previously (19). These compounds were not present in liver lipids from rats fed unheated linseed oil (24). The first one was identified some years ago as the 20:5 Δ5*cis*, 8*cis*, 11*cis*, 14*cis*, 17*trans* isomer of EPA (12), which was formed from 18:3 Δ9*cis*, 12*cis*, 15*trans*. Using the DB Wax column, EPA itself and four unknown peaks, which were called X1, X2, X3, and X4, are then eluted. Their respective equivalent chain length (ECL) values are presented in Table 1. The GLC–FTIR spectra obtained for X4 (Fig. 1) showed an absorption band at 966 cm^{-1}, which is characteristic of a *trans* ethylenic bond (25). X3 presented a similar FTIR spectrum, indicating the occurrence of at least one *trans* double bond on the carbon chain.

Figure 2 presents the mass spectra of the DMOX derivatives of EPA, X3, and X4. The fragmentation of EPA is the same as that described previously (23). Moreover, the three fragmentation patterns were similar, indicating that X3 and X4 are also 20:5 fatty acids, with their double bonds located at the Δ5, 8, 11, 14, and 17 positions (25). These gas-chromatographic–FTIR and gas chromatography/mass spectrometry results clearly indicate that X3 and X4 are *trans* isomers of EPA. Furthermore, comparisons of the ECL values on both types of liquid phase and the fragmentation pattern of X3 and X4 with those of the synthetized compounds (20) confirmed that X3 and X4 are 20:5 Δ5*cis*, 8*cis*, 11*trans*, 14*cis*, 17*trans*, and 20:5 Δ5*cis*, 8*cis*, 11*trans*, 14*cis*, 17*cis*, respectively.

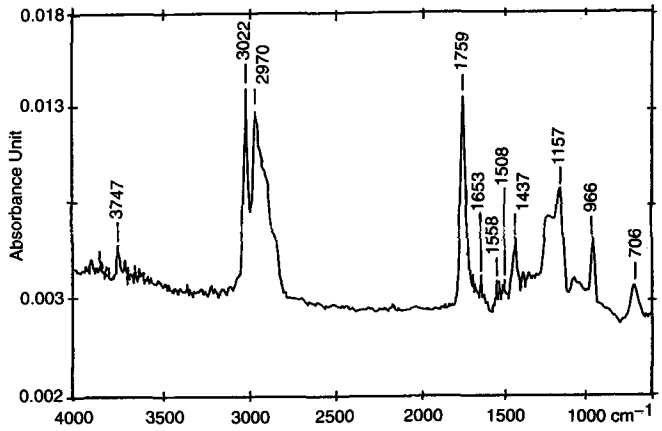


FIG. 1. Infrared spectrum of compound X4 obtained by gas-chromatographic–Fourier transform infrared. The chromatographic conditions are described in the Materials and Methods section.

Table 2 indicates the mean level of each 20:5 *trans* isomer in liver lipids of five rats fed heated oil for six weeks. Under these dietary conditions, the major 20:5 fatty acids were the Δ17 mono*trans* isomer and EPA (more than 80% of total 20:5 fatty acids). The 20:5 Δ11,17 di*trans* and Δ11 mono*trans* isomers represented about 5% of total 20:5 fatty acids. These are the desaturation and elongation metabolites of 18:3 Δ9*trans*, 12*cis*, 15*trans*, and of 18:3 Δ9*trans*, 12*cis*, 15*cis*, respectively. Both of these 18:3 isomers are present at significant levels in dietary oils (1,2,4,26). X1 and X2 might be other 20:5 *trans* fatty acids as suggested by Piconneaux (24). If so, they would be the metabolites of other 18:3 isomers (e.g., Δ9*cis*, 12*trans*, 15*trans*, and Δ9*cis*, 12*trans*, 15*cis*), which are minor constituents of some processed vegetable oils. Consequently, X1 and X2 are not of major human nutritional concern. Table 3 indicates the ratio between the 18:3 *trans* isomers in the diet and their respective 20:5 metabolites in the liver lipids of rats. These ratios probably indicate the rate of conversion of each *trans* 18:3 isomer into the corresponding *trans* 20:5 fatty acid. Both Δ9*cis* 18:3 isomers (Δ9*cis*, 12*cis*, 15*cis*, and Δ9*cis*, 12*cis*, 15*trans*) presented an accumulation (18:3/20:5) of about four. This might be related to the occurrence of the *cis* double bond at the Δ9 position, which was reported as helpful for the Δ6 desaturation (27). Both Δ9 *trans* isomers presented a lower rate of accumulation. However, the difference between the propensity for these two fatty acids (20.0 vs. 7.6) to accumulate is not clearly understood. The respective influ-

TABLE 1
ECL of the Six Fatty Acid Methyl Esters from the 20:5 Fraction Isolated from Liver Lipids of Rats Fed a Heated Linseed Oil[a]

	DB Wax (190°C)	BPX 70 (170°C)
Eicosapentaenoic acid	21.78	22.00
20:5 Δ5*cis*, 8*cis*, 11*cis*, 14*cis*, 17*trans*	21.72	21.72
X1	21.84	21.56
X2	21.96	21.92
X3	22.04	21.84
X4	22.12	22.16

[a]Equivalent chain length (ECL) were calculated from analyses performed on a DB Wax (J&W Scientific, Rancho Cordo, CA) and a BPX 70 capillary columns (SGE, Melbourne, Australia).

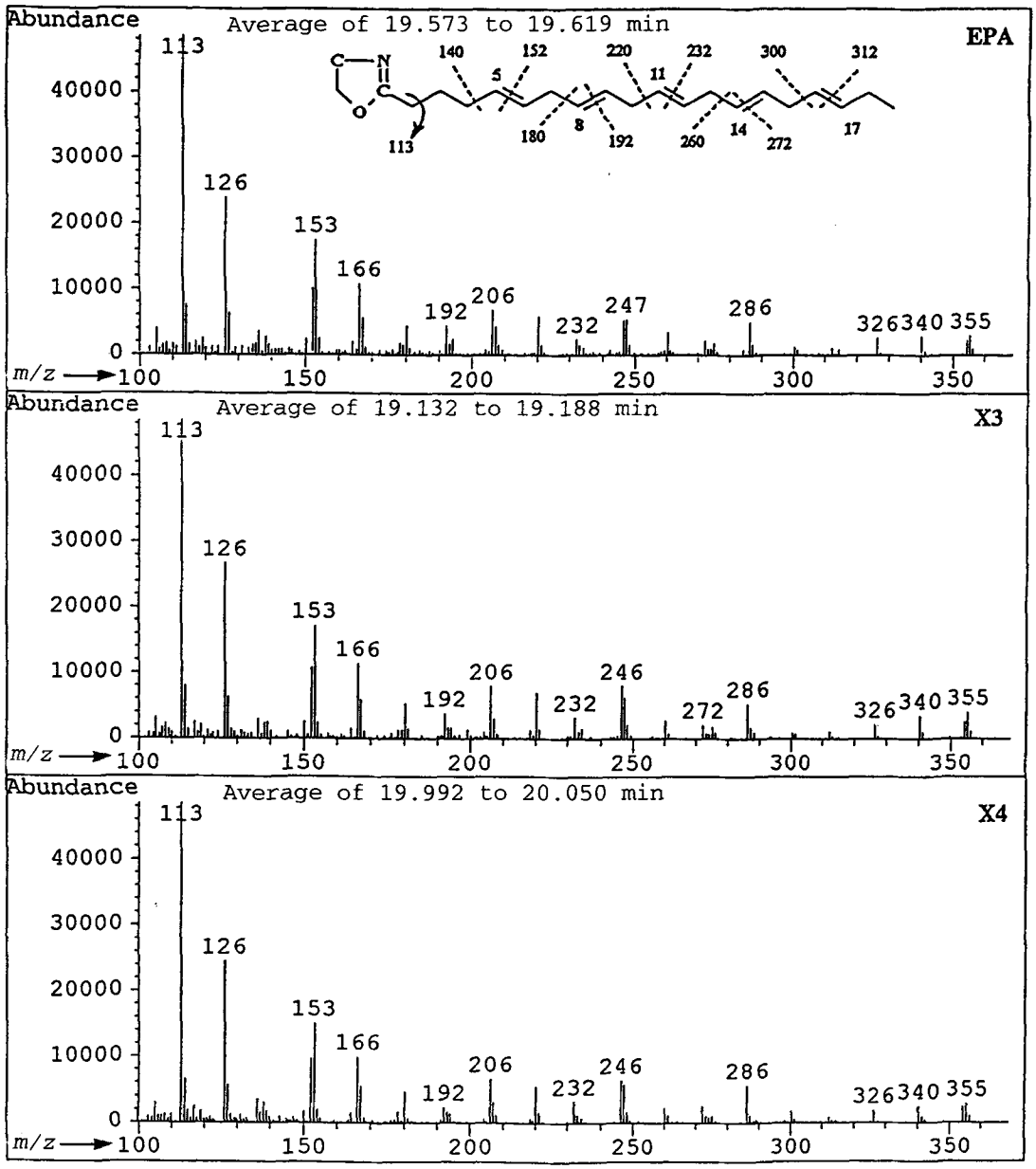


FIG. 2. Mass spectra of dimethyl oxazoline derivatives of eicosapentaenoic acid (EPA), X3, and X4. The chromatographic conditions are described in the Materials and Methods section.

TABLE 2
Percentage of 20:5 Isomers (related to total 20:5 fatty acids) of Liver Lipids from Rats Fed 10% of Heated Linseed Oil[a]

	20:5 isomers					
	EPA	20:5 Δ17*trans*	X1	X2	X3	X4
% of total FAME	0.25 ± 0.010	0.95 ± 0.099	0.12 ± 0.006	0.06 ± 0.006	0.04 ± 0.006	0.03 ± 0.005
% of total 20:5	17.2	65.5	8.3	4.1	2.8	2.1

[a]The mean liver weight was 17.4 ± 0.3 g, and the liver lipid content was 1.86 ± 0.17 g (means ± SEM of five determinations); EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester.

TABLE 3
Ratios (× 100) of the Quantity of 18:3 Isomers in the Diet to 20:5 Isomers in the Liver Lipids

18:3/20:5	Ratio
18:3 Δ9*cis*, 12*cis*, 15*cis*/20:5 Δ5*cis*, 8*cis*, 11*cis*, 14*cis*, 17*cis*,	4.1
18:3 Δ9*cis*, 12*cis*, 15*trans*/20:5 Δ5*cis*, 8*cis*, 11*cis*, 14*cis*, 17*trans*	4.3
18:3 Δ9*trans*, 12*cis*, 15*cis*/20:5 Δ5*cis*, 8*cis*, 11*trans*, 14*cis*, 17*cis*	20.0
18:3 Δ9*trans*, 12*cis*, 15*trans*/20:5 Δ5*cis*, 8*cis*, 11*trans*, 14*cis*, 17*trans*	7.6

ence of each *trans* double bond on the acylation and further metabolism of these fatty acids requires further work.

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Chronic Administration of Eicosapentaenoic Acid and Docosahexaenoic Acid as Ethyl Esters Reduced Plasma Cholesterol and Changed the Fatty Acid Composition in Rat Blood and Organs

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ABSTRACT: Fish oils rich in n-3 fatty acids have been shown to decrease plasma lipid levels, but the underlying mechanism has not yet been elucidated. This investigation was performed in order to further clarify the effects of purified ethyl esters of eicosapentaenoic acid (EPA–EE) and docosahexaenoic acid (DHA–EE) on lipid metabolism in rats. The animals were fed EPA–EE, DHA–EE, palmitic acid, or corn oil (1 g/kg/d) by orogastric intubation along with a chow background diet for three months. At the end the animals were sacrificed. Plasma and liver lipids were measured, as well as lipid-related enzyme activities and mRNA levels. The fatty acid composition of plasma and different tissues was also determined. This study shows that, compared to the corn oil control, EPA–EE and DHA–EE lowered plasma cholesterol level, whereas only EPA–EE lowered the amount of plasma triacylglycerol. In liver peroxisomes, both EE preparations increased fatty acyl-CoA oxidase FAO activities, and neither altered 3-hydroxy-3-methylglutaryl (HMG)–CoA reductase activities. In liver microsomes, EPA–EE raised HMG–CoA reductase and acyl-CoAicholesterol acyltransferase activities, whereas DHA–EE lowered the former and did not affect the latter. Neither product altered mRNA levels for HMG–CoA reductase, low density lipoprotein-receptor, or low density lipoprotein-receptor related protein. EPA–EE lowered plasma triacylglycerol, reflecting lowered very low density lipoprotein secretion, thus the cholesterol lowering effect in EPA–EE-treated rats may be secondary to the hypotriacylglycerolemic effect. An inhibition of HMG–CoA reductase activity in DHA–EE treated rats may contribute to the hypocholesterolemic effect. The present study reports that 20:5n-3, and not 22:6n-3, is the fatty acid primarily responsible for the triacylglycerol lowering effect of fish oil. Finally, 20:5n-3 was not converted to 22:6n-3, whereas retroconversion of 22:6n-3 to 20:5n-3 was observed.
Lipids 31, 169–178 (1996).

The hypotriacylglycerolemic effect of marine oils containing very long-chain n-3 fatty acids, such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), has been extensively studied in humans and in different animal species and cultured cells (1–6). Fish oil lowers plasma triacylglycerol by reducing the hepatic production and secretion of lipoprotein particles rich in triacylglycerol, e.g., very low density lipoprotein (VLDL) (7–13) and by inhibiting the last enzyme in triacylglycerol biosynthesis, acyl-CoA:diacylglycerol acetyltransferase in rats (14). In addition to the effect on VLDL, fish oil affects nearly every major pathway of hepatic fatty acid metabolism (15,16). Evidence from *in vivo* and *in vitro* experiments in rats indicates that the instant hypotriacylglycerolemic effect observed after 20:5n-3 and fish oil administration could be attributed, at least in part, to a sudden increase in mitochondrial fatty acid oxidation (17–19). This may reduce the availability of fatty acids for lipid synthesis in the liver for export, e.g., in the form of VLDL (20). The changes of acyl-CoA:diacylglycerol acetyltransferase, however, could not explain the reduced availability of fatty acids for triacylglycerol biosynthesis (15,21). Recently, our group has reported (22,23) that 20:5n-3 and 22:6n-3 possess different metabolic properties, i.e., no triacylglycerol or cholesterol-lowering effects were observed in rats treated acutely and for up to 15 d with 22:6n-3, whereas 20:5n-3 decreased plasma cholesterol and triacylglycerol levels. These results are in agreement with results obtained by the isolated perfused rat liver (24).

The mechanism behind the cholesterol-lowering effect of polyunsaturated fat is at present not known in detail. n-3 Fatty acids have structural roles in glycerolipids of all cell membranes in the body, influencing membrane viscosity and permeability, and thereby possibly affecting the enzyme activity of membrane proteins. It has been reported that the hypocholesterolemic effect of fish oil feeding is accompanied by a significant inhibition of the microsomal 3-hydroxy-3-methylglutaryl–CoA (HMG–CoA) reductase activity (17).

The rate-limiting enzyme in cholesterol formation, HMG–CoA reductase, is localized in the endoplasmic reticu-

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Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; DHA, docosahexaenoic acid; DHA–EE, DHA as ethyl ester; EPA, eicosapentaenoic acid; EPA–EE, EPA as ethyl ester; HMG–CoA, 3-hydroxy-3-methylglutaryl–CoA; LDL, low density lipoprotein; LRP-4, LDL-receptor related protein; VLDL, very low density lipoprotein.

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lum and in intact peroxisomes (25,26). Both EPA–EE [EPA as ethyl ester (EE)] and DHA–EE [DHA as ethyl ester (EE)] treatment increased peroxisomal β-oxidation in the liver, as evaluated by measuring fatty acyl-CoA oxidase activity (FAO) (9,15,16). These observations led us to study the peroxisomal HMG–CoA reductase activity after long-term administration of EPA–EE and DHA–EE.

The degree of unsaturation of the fatty acyl chains may affect the fluidity of the lipid moiety of lipoproteins. Indeed, it has been suggested that polyunsaturated fat feeding may influence the structure of the low density lipoprotein (LDL) particle by changing the fluidity of its constituent lipids and thereby influencing the rate at which the particles can be catabolized (27).

The present study was conducted with a threefold purpose. First, we wanted to investigate whether DHA–EE and EPA–EE have a cholesterol-lowering effect after prolonged administration. Second, we addressed the question of whether cholesterol biosynthesis is regulated by the peroxisomal HMG–CoA reductase or by changes in the expression of LDL-receptor and LDL-receptor related protein (LRP-4) as denoted by their relative mRNA levels, both of which are thought to be important for the final catabolism of LDL and chylomicron remnants (28), respectively, by the liver. LRP-4 is composed completely of structural elements found in the LDL-receptor (29). Finally, we studied whether conversion of 20:5n-3 to 22:6n-3 and retroconversion of 22:6n-3 to 20:5n-3 occurs in rat plasma and organs (liver, heart, kidney, and epididymal fat) after prolonged feeding with EPA–EE and DHA–EE.

MATERIALS AND METHODS

Chemicals. EPA–EE and DHA–EE were obtained from Pronova Biocare AS (Oslo, Norway) and Norsk Hydro AS, Research Center (Porsgrunn, Norway). The purity of EPA–EE and DHA–EE used in the present study was 94 and 91%, respectively (Table 1). EPA–EE contained small amounts of stearidonic acid (18:4n-3) (1.1%) and DHA (22:6n-3) (1.8%), and the DHA–EE preparation contained some arachidonic acid (20:4n-6) (1.9%), eicosatetraenoic acid (20:4n-3) (1.0%), EPA (20:5n-3) (2.3%), and heneicosapentaenoic acid (21:5n-3) (4.6%). Corn oil was bought at the local store, and it contained linoleic acid (18:2n-6) (51.3%), oleic acid (18:1n-9), (33.6%), and palmitic acid (16:0) (10.1%).

Palmitic acid was obtained from Sigma Chemical Co. (St. Louis, MO). [1-^{14}C]Palmitic acid (56 mCi/mmol), [^{14}C]acetyl-CoA (60 mCi/mmol), and $NaH^{14}CO_3$ (0.1 mCi/mmol) were purchased from the Radiochemical Centre (Amersham, England). [4-^{14}C]Cholesterol and [3-^{14}C]hydroxymethylglutaryl–CoA, with specific activities ranging from 55–60 mCi/mmol, were obtained from New England Nuclear (Boston, MA). All other chemicals were obtained from common commercial sources and were of reagent grade.

Animals. Male Wistar rats from Möllegaard Breeding Laboratory (Ejby, Denmark), weighing about 200 g, were housed

TABLE 1
Fatty Acid Composition of the Total Lipid of the Experimental Fatty Acids and the Standard Chow (R34-EWOS-ALAB)[a]

Fatty acids	Palmitic acid	EPA–EE	DHA–EE	Corn oil	R34-EWOS-ALAB
16:0	96.8 ± 3.1	0.1 ± 0.1	0.5 ± 0.5	10.0 ± 0.0	21.0 ± 0.2
16:1n-7					2.0 ± 0.1
16:1n9		0.2 ± 0.2		0.1 ± 0.0	
16:2n-4	0.1 ± 0.1	0.1 ± 0.0			
17:0		0.2 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	
18:0	0.2 ± 0.0		0.1 ± 0.1	1.7 ± 0.0	4.0 ± 0.1
18:1n-7		0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.0	
18:1n-9		0.2 ± 0.0	0.3 ± 0.3	33.6 ± 0.1	25.0 ± 0.1
18:2n-6	0.1 ± 0.1	0.4 ± 0.2	0.1 ± 0.1	51.3 ± 0.1	42.0 ± 0.4
18:3n-3		0.1 ± 0.1	0.3 ± 0.1	1.5 ± 0.1	6.0 ± 0.1
18:4n-4		1.1 ± 0.1	0.1 ± 0.0		
20:0				0.4 ± 0.1	
10:1n-n-6				0.3 ± 0.1	
10:1n-7			0.1 ± 0.1		
20:3n-6		0.1 ± 0.1			
20:4n-3			1.0 ± 0.3		
20:4n-6		0.2 ± 0.1	1.9 ± 0.2		
20:5n-3	0.1 ± 0.1	93.8 ± 1.9	2.3 ± 0.2		
21:5n-3			4.6 ± 0.2		
22:5n-3		0.3 ± 0.1	0.1 ± 0.0		
22:6n-3		1.8 ± 0.3	91.2 ± 2.3		
Unknown		0.3 ± 0.1	0.5 ± 0.1		

[a]Values are given as mean ± SD. Composition of diets (mol%). EPA–EE, eicosapentaenoic acid as ethyl ester; DHA–EE, docosahexaenoic acid as ethyl ester; R34-EWOS-ALAB, grover rat maintenance chow.

individually in metal wire cages and maintained at a 12-h light/dark cycle, at a temperature of 20 ± 3°C. The animals were acclimatized for one week under these conditions before the start of the experiment. The fatty acids were suspended in 0.5% CM-cellulose and administered by orogastric intubation once a day for three months at a dose of 1000 mg/d/kg body weight. Autooxidation of the stock suspensions was minimized by the addition of 0.5% (wt/vol) of α-tocopherol (vitamin E), flushing with N_2, and storing them at 0–4°C when not in use. All animals had free access to water and R34-EWOS-ALAB (12.5 MJ/kg) (Ewos, Sweden) grower rat maintenance chow. At the end of the experiment, the overnight-fasted (to avoid postprandrial values) animals were anaesthetized with Hypnorm (Janssen Pharmaceutica BV, Beerse, Belgium) Dormicum® (F. Hoffman-La-Roche Ltd., Basel, Switzerland) (fluanisone-fentanyl-midazolam) subcutaneously, and cardiac puncture was performed to obtain blood samples in EDTA (7.5%) containing vacutainers. Plasma was prepared from whole blood by centrifugation at 3000 rpm (1880 × *g*) for 10 min. The protocol was approved by the Norwegian State Board of Biological Experiments with Living Animals.

Preparation of subcellular fractions. The livers from individual rats were removed, weighed, and immediately chilled on ice. Portions of the livers were homogenized in ice-cold sucrose medium (0.25 M sucrose in 10 mM HEPES buffer and 1 mM EDTA, pH 7.4). Peroxisomal-enriched and microsomal fractions were prepared as previously described (15,16,22).

Enzyme assays. HMG–CoA reductase activity was assayed in the peroxisomal and microsomal fractions essentially as previously described (30). The samples were incubated for 15 min and terminated by addition of 25 µL 6 M HCl. Tritium-labeled mevalonic acid (0.01 µCi), together with 3 mg unlabelled mevalonic acid lactone, was added as internal standard. The incubation mixture was further lactonized, subjected to thin-layer chromatography, and analyzed for radioactivity (17).

Acyl-CoA:cholesterol acyltransferase (AcAT) activity was measured in the microsomal fraction (31), and activity was determined as previously described (15).

Analytical methods. Plasma free fatty acids were determined by an enzymatic colorimetric method (NEFAC; Wako Chemicals GmbH) as described previously (32). Protein was determined spectrophotometrically at 600 nm by the pyrogallol red-molybdate method (33) due to low protein concentrations. Phospholipids and cholesterol were analyzed using the enzymatic methods of bioMérieux (Marcy-l'Etoile, France) and Boehringer Mannheim (Mannheim, Germany), respectively.

Analysis of hepatic lipids was carried out by the Monotest cholesterol and phospholipid enzymatic kits (Boehringer Mannheim) and the Biopak triglyceride enzymatic kit (Biotrol, Paris, France). Protein was determined by the Bio-Rad assay kit (Bio-Rad Laboratories, Richmond, CA).

RNA purification. Livers were frozen in liquid N_2 immediately after dissection and stored at –80°C. Total cellular RNA was purified using the acid guanidinium thiocyanate-phenol method (34). The RNA concentrations were determined by measuring the ultraviolet absorbance at 260 nm.

Preparation of hybridization probes. The appropriate DNA fragments were excised from plasmids by restriction enzymes, and the purified fragments were ^{32}P-labeled using the oligo-labeling technique (35). The resulting specific activities ranged from $1–5 \times 10^9$ cpm/µg. The probes were purified fragments of cloned genes: rat LDL-receptor; 2.2 bp *Eco* RI inserted into pGEM7 (36), HMG–CoA reductase cDNA from baby hamster kidney cells; full-length *Eco* RI fragment of pDGS2, rat LRP-4; *Bam* HI fragment of a 3.6 kb cDNA inserted into pGEM4 (29), 28S rRNA; 1.4 kb *Bam* HI fragment of pA.

Hybridization and analysis of mRNA. Slot blotting of RNA onto nylon membranes was carried out as previously described (37). Hybridization to immobilized RNA was carried out in the presence of 50% formamide, 5 × SSC (0.75 M NaCl, 75 mM trisodium citrate dihydrate), 200 µg/mL heat-denaturated herring sperm DNA, 0.1% SDS, 25 µM sodium phosphate, (pH 6.4), 8.25% dextran sulphate at 42°C for 24–48 h (38). Filters were stringently washed twice in 0.2 × SSC, 0.1% SDS, 0.1% $Na_4P_2O_7$ at 65°C (or at 60°C for DNA probes not obtained from rats). When the filters were to be rehybridized, the bound probe was first stripped off by incubation of the filters in 0.1% SDS at 95–100°C for 7 min.

Kodak XAR 5 X-ray films were exposed to the membranes at –80°C in the presence of intensifying screens. Autoradiograms were analyzed by densiometric scanning using an LKB Ultrogel laser densiometer (Bromma, Sweden). The relative level of mRNA expression was estimated as the amount of radioactive probe hybridized to each sample of RNA relative to the level of 28S rRNA in each sample.

Statistical analysis. Results are reported as means ± SD from six animals. Treatment means were compared by one-way analysis of variance. Fisher's Least Square Difference was used to determine the significance of differences between the means at 95% confidence interval.

RESULTS

There were no significant changes in the body weight between the rats fed corn oil (control) and those of the other groups at the end of the experiment (data not shown).

As shown in Table 2, prolonged administration of EPA–EE to rats (maintained on a balanced pelleted diet) resulted in a lowering of plasma triacylglycerol, cholesterol, and phospholipid levels compared to corn oil-fed animals. Prolonged DHA–EE treatment did not affect the plasma triacylglycerol level but significantly lowered the concentration of plasma cholesterol as compared to control animals (Table 2). The plasma free fatty acids content (Table 2) and the hepatic triacylglycerol and phospholipid levels (Table 3) were not significantly changed after EPA–EE and DHA–EE treatment. The hepatic cholesterol concentration in EPA–EE treated rats was significantly higher than in controls (Table 3).

TABLE 2
Changes in Plasma Lipids in Rats After Prolonged Treatment (3 mon) with Palmitic Acid, EPA–EE, DHA–EE, and Corn Oil (control)[a]

Plasma lipids (mM)	Control	EPA–EE	DHA–EE	Palmitic acid
Triacylglycerol	1.81 ± 0.47[c]	1.05 ± 0.46[b]	1.74 ± 0.60[c]	1.51 ± 0.30[c]
Cholesterol	1.65 ± 0.22[c]	0.95 ± 0.14[b]	1.08 ± 0.23[b]	1.52 ± 0.37[c]
Phospholipids	1.71 ± 0.24[c]	1.13 ± 0.13[b]	1.44 ± 0.20[c]	1.54 ± 0.23[c]
Free fatty acids	0.46 ± 0.24[c]	0.42 ± 0.12[c]	0.34 ± 0.13[c]	0.56 ± 0.19[c]

[a]The plasma lipid values represent means ± SD from six animals in each experimental group. Means in a row with a different superscript are significantly different ($P < 0.05$). Abbreviations as in Table 1.

TABLE 3
The Effect of Prolonged (3 mon) EPA–EE, DHA–EE, Palmitic Acid, and Corn Oil (control) Feeding on Hepatic Lipids[a]

Hepatic lipids (μmol/g liver)	Control	EPA–EE	DHA–EE	Palmitic acid
Triacylglycerol	8.08 ± 1.54[c]	7.65 ± 1.55[c]	6.80 ± 1.52[c]	6.00 ± 1.00[c]
Cholesterol	3.20 ± 0.38[c]	4.20 ± 0.50[b]	3.36 ± 0.25[c]	2.91 ± 0.26[c]
Phospholipids	11.90 ± 1.39[c]	12.56 ± 1.49[c]	12.10 ± 0.90[c]	11.72 ± 0.70[c]

[a]The plasma lipid values represent means ± SD from six animals in each experimental group. Means in a row with a different superscript are significantly different ($P < 0.05$). Abbreviations as in Table 1.

Peroxisomal HMG–CoA reductase activity remained unchanged after EPA–EE and DHA–EE feeding, whereas the activity of the microsomal HMG–CoA reductase was higher and lower after EPA–EE and DHA–EE feeding, respectively, compared to control animals (Table 4). Rats treated with EPA–EE and DHA–EE did not affect HMG–CoA reductase mRNA levels as compared to control, whereas palmitic acid-fed animals revealed a higher HMG–CoA reductase mRNA level (Fig. 1 and Table 5). The activity of ACAT was higher after EPA–EE feeding compared to corn oil-fed animals (Table 4), whereas no change was observed in DHA–EE–treated rats.

When rats were treated with EPA–EE and DHA–EE, no evidence was found for lower LDL-receptor mRNA levels compared to control animals (Table 5). Moreover, the LRP-4 mRNA levels were constant in the different feeding regimes (Table 5).

Rats treated with EPA–EE, DHA–EE, and palmitic acid revealed higher levels of palmitic acid (16:0) and total saturated fatty acids in epididymal fat (Table 6) and lower levels of linoleic acid (18:2n-6) compared to controls (Table 7). In addition, EPA–EE and DHA–EE lowered the amount of oleic acid (18:1n-9) and total monoenes in epididymal fat as compared to corn oil-fed animals (Table 6). No such changes were observed in plasma, liver, heart, and kidney, except that the total saturated fatty acids was higher in plasma after palmitic acid administration, and EPA–EE treatment lowered the amount of vaccenic acid (18:1n-7) compared to controls (Table 6).

As shown in Table 7, DHA–EE and palmitic acid treatment lowered the amount of 18:2n-6 in the heart, whereas

TABLE 4
The Effect of Prolonged (3 mon) EPA–EE, DHA–EE, Palmitic Acid, and Corn Oil (control) Feeding on the Rate-Limiting Enzyme in Peroxisomal β-Oxidation of Fatty Acids and on Enzymes in Cholesterol Metabolism[a]

Enzymes	Control	EPA-EE	DHA-EE	Palmitic acid
Peroxisomes				
FAO				
(nmol/min/mg protein)	20.3 ± 2.6[c]	29.5 ± 2.1[b]	30.5 ± 4.2[b]	18.3 ± 2.5[c]
HMG–CoA reductase				
(pmol/min/mg protein)	5.4 ± 1.2[c]	5.3 ± 1.0[c]	5.6 ± 1.3[c]	5.5 ± 1.8[c]
Microsomes				
HMG–CoA reductase				
(nmol/min/mg protein)	1.8 ± 0.3[c]	2.2 ± 0.3[b]	1.0 ± 0.1[d]	1.4 ± 0.3[c]
ACAT				
(pmol/min/mg protein)	147.4 ± 31.6[c]	219.8 ± 34.2[b]	165.4 ± 55.8[c]	135.4 ± 32.1[c]

[a]The values are given as means ± SD. Means in a row with a different superscript are significantly different ($P < 0.05$). Abbreviations as in Table 1; FAO, fatty acyl-CoA oxidase; HMG–CoA, 3-hydroxy-3-methylglutaryl–CoA; ACAT, acyl-CoA:cholesterol acyltransferase).

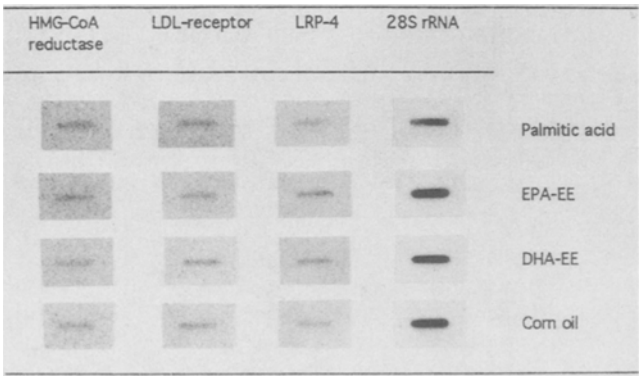


FIG. 1. mRNA expression of 3-hydroxy-3-methylglutaryl–CoA (HMG–CoA) reductase, low density lipoprotein (LDL)-receptor, LDL-receptor related protein (LRP-4), and 28S rRNA in liver of rats treated with eicosapentaenoic acid as ethyl ester (EPA–EE), docosahexaenoic acid as ethyl ester (DHA–EE), palmitic acid, and corn oil (control) for three months. One representative slot blot of a total of six is shown. The accumulated results from a complete experiment are given in Table 6. Total RNA was isolated from liver pieces of each animal in the different feeding groups. Samples of 1, 2, and 5 μg RNA were slot-blotted onto nylon membranes and hybridized to the probes indicated (see the Materials and Methods section).

EPA–EE treatment led to a higher level of α-linolenic acid (18:3n-3) in the kidney as compared to controls (Table 7).

EPA–EE and DHA–EE supplementation significantly lowered the amount of arachidonic acid (20:4n-6) in plasma, liver, heart, and kidney as compared to control animals, which led to a higher n-3/n-6 ratio in plasma and the different organs (Table 7). No significant differences in n-3/n-6 ratio between the two n-3 fatty acids were observed.

As shown in Table 7, prolonged EPA–EE feeding led to a higher amount of EPA (20:5n-3) in plasma, liver, heart, kidney, and epididymal fat by 11-, 17-, 36-, 11-, and 41-fold, respectively, as compared to controls. The amount of docosapentaenoic acid (22:5n-3) was also higher in plasma, liver, heart, kidney, and epididymal fat, by 5-, 5-, 4-, 5-, and 8-fold, respectively (Table 7). However, the levels of DHA (22:6n-3) were not higher after EPA–EE treatment, except for a 2-fold increase in epididymal fat (Table 7). DHA–EE treatment caused a less prominent elevation of DHA (22:6n-3) in plasma and organs than EPA–EE feeding, which increased 20:5n-3. The rise of 22:6n-3 levels by DHA–EE administration in plasma, liver, heart, kidney, and epididymal fat was 4-, 3-, 2-, 3-, and 11-fold, respectively, whereas a higher level of 22:5n-3 was only observed in kidney and epididymal fat.

It was noteworthy that, DHA–EE supplementation elevated the amount of 20:5n-3 in plasma, liver, heart, kidney, and epididymal fat by 5-, 8-, 6-, 7-, and 7-fold, respectively.

DISCUSSION

Plasma concentrations of EPA (20:5n-3) and DHA (22:6n-3) were significantly increased after pure EPA–EE (94%) and pure DHA–EE (91%) administration. The elongated product of 20:5n-3, docosapentaenoic acid (22:5n-3) was higher in plasma after EPA–EE administration compared to corn oil-treated rats, whereas no increased amount of 22:6n-3 was revealed. In the plasma of DHA–EE treated rats, however, 22:5n-3 remained unchanged, but the relative amount of 20:5n-3 was increased. Thus, DHA–EE treatment caused a less prominent increase in 22:6n-3 in the DHA–EE group than EPA–EE feeding, increased the amount of 20:5n-3 in the EPA–EE group in plasma and organs, respectively (Table 7). These results suggest that conversion of 20:5n-3 to 22:5n-3, but not to 22:6n-3, and retroconversion of 22:6n-3 to 20:5n-3 have occurred in tissues after n-3 fatty acid administration, as demonstrated in cultured hepatocytes (39–41).

The underlying assumption has long been that 20:5n-3 is elongated and desaturated directly to 22:6n-3. Recently Voss *et al.* (42) have shown in the rat that, although 20:5n-3 can be converted into 22:6n-3, it has to go through three steps, namely: first, being elongated to 24:5n-3-, second, Δ^6-desaturated to 24:6n-3; and finally, β-oxidized to 22:6n-3 (42). The results of our study argue against a direct conversion of 20:5n-3 to 22:6n-3 as the level of 22:6n-3 was unchanged in the liver, heart, and kidney after EPA–EE administration. As the amount of 22:5n-3 was increased in plasma and organs after EPA–EE supplementation (Table 7) and the peroxisomal β-oxidation was increased (Table 4), the unchanged level of 22:6n-3 could be due to a depressed Δ^6-saturation. Indeed, di-

TABLE 5
Expression of HMG–CoA Reductase, LDL-Receptor, and LRP-4 mRNAs in Liver of Rat Treated with Palmitic Acid, EPA–EE, DHA–EE, and Corn Oil (control) for Three Months[a]

Treatment	HMG–CoA reductase	LDL-receptor	LRP-4
Control	1.00 ± 0.10[c]	1.00 ± 0.20[c]	1.00 ± 0.17[c]
Palmitic acid	1.40 ± 0.13[b]	1.03 ± 0.27[c]	1.21 ± 0.37[c]
EPA–EE	0.90 ± 0.16[c]	0.85 ± 0.14[c]	1.24 ± 0.34[c]
DHA-EE	0.95 ± 0.16[c]	0.83 ± 0.24[c]	1.17 ± 0.25[c]

[a]The table shows the relative mRNA levels of HMG–CoA reductase, low density lipoprotein (LDL)-receptor, and (LRP-4), as measured by densiometric scanning of the autoradiograms. The relative expression of mRNA levels was normalized to the corresponding 28S rRNA levels (i.e., the ratio of mRNA/28S rRNA), means of controls set to 1.00. Values are presented as means ± SD. Means in a column with a different superscript are significantly different ($P < 0.05$). Abbreviations as in Tables 1 and 4.

TABLE 6
Changes in Fatty Acid Composition in Plasma and Different Tissues of Rats Treated with EPA–EE, DHA–EE, Palmitic Acid (palm), and Corn Oil (corn) (control) for Three Months[a]

Fatty acid	Treatment	Fatty acid composition (mol%)				
		Plasma	Liver	Heart	Kidney	Epididymal fat
16:0	Corn	21.5 ± 1.2	22.2 ± 0.7	12.9 ± 2.2	21.5 ± 1.3	22.5 ± 0.8
	EPA–EE	21.7 ± 2.0	22.2 ± 0.9	13.2 ± 2.1	25.8 ± 2.4	24.7 ± 2.4[b]
	DHA–EE	22.4 ± 2.3	23.1 ± 1.6	18.7 ± 6.4	23.5 ± 0.8	25.6 ± 2.0[b]
	Palm	25.5 ± 3.1	22.5 ± 1.4	17.9 ± 4.8	23.0 ± 1.5	27.2 ± 1.3[b]
18:0	Corn	4.2 ± 0.6	15.1 ± 0.9	17.7 ± 3.0	15.2 ± 1.6	2.6 ± 0.1
	EPA–EE	5.2 ± 0.8	14.2 ± 1.7	18.1 ± 1.9	13.3 ± 3.1	3.0 ± 0.3
	DHA–EE	4.8 ± 1.2	15.1 ± 1.1	13.9 ± 5.1	14.4 ± 2.8	3.0 ± 0.2
	Palm	5.1 ± 0.8	15.5 ± 3.0	15.1 ± 3.5	13.9 ± 1.9	2.8 ± 0.6
Total saturated	Corn	28.0 ± 1.0	42.2 ± 1.7	31.5 ± 0.5	38.1 ± 2.7	26.9 ± 0.8
	EPA–EE	28.9 ± 2.8	38.9 ± 1.7	32.3 ± 0.6	40.8 ± 4.2	29.7 ± 2.6[b]
	DHA–EE	28.1 ± 2.4	43.0 ± 3.2	34.2 ± 2.1	39.4 ± 2.5	30.7 ± 1.8[b]
	Palm	33.7 ± 3.5[a]	41.5 ± 3.9	34.3 ± 2.1	38.4 ± 1.4	32.0 ± 0.9[b]
16:1n-7	Corn	4.1 ± 1.2	2.2 ± 0.6	0.8 ± 0.6	2.2 ± 0.4	5.1 ± 1.0
	EPA–EE	3.5 ± 0.9	2.2 ± 0.6	0.8 ± 0.6	3.8 ± 0.7	5.8 ± 1.6
	DHA–EE	4.4 ± 2.1	2.4 ± 1.1	2.7 ± 2.4	3.2 ± 1.1	5.8 ± 1.6
	Palm	4.1 ± 2.2	2.3 ± 1.4	1.9 ± 1.6	3.0 ± 0.6	7.0 ± 1.9
18:1n-7	Corn	3.9 ± 0.9	4.6 ± 0.9	3.4 ± 0.3	3.2 ± 0.2	4.2 ± 0.4
	EPA–EE	2.2 ± 0.4[a]	3.2 ± 0.3	3.2 ± 0.2	2.2 ± 0.3	3.7 ± 0.3
	DHA–EE	2.7 ± 0.7	3.2 ± 0.7	3.1 ± 0.4	2.2 ± 0.3	3.9 ± 0.4
	Palm	4.2 ± 1.2	4.4 ± 0.8	3.6 ± 0.4	2.8 ± 0.2	5.1 ± 0.4[b]
18:1n-9	Corn	14.4 ± 1.2	6.6 ± 1.0	6.7 ± 4.4	12.7 ± 1.3	24.4 ± 1.0
	EPA–EE	14.8 ± 2.3	7.3 ± 1.5	5.8 ± 3.0	14.5 ± 2.8	19.7 ± 1.5[b]
	DHA–EE	13.9 ± 1.7	6.1 ± 0.9	12.2 ± 9.8	12.3 ± 2.7	19.1 ± 0.9[b]
	Palm	15.2 ± 4.5	6.4 ± 1.8	9.2 ± 6.2	13.3 ± 2.3	22.7 ± 0.2
Total monoenes	Corn	23.5 ± 3.0	14.1 ± 2.1	11.3 ± 5.2	17.8 ± 1.7	35.0 ± 1.6
	EPA–EE	22.6 ± 3.7	12.4 ± 2.1	10.3 ± 3.6	21.1 ± 3.8	30.5 ± 2.2[b]
	DHA–EE	19.4 ± 9.8	12.4 ± 2.5	18.6 ± 12.4	18.1 ± 4.1	30.2 ± 2.7[b]
	Palm	24.2 ± 7.9	13.1 ± 4.0	15.4 ± 7.9	19.6 ± 2.9	35.9 ± 1.1

[a]Values are expressed as means ± SD. Abbreviations as in Table 1.
[b]Significantly different from the control at $P < 0.05$.

etary supplementation with n-3 fatty acids depresses rat liver Δ^6-desaturase activity (43), which is necessary for the second step of the conversion of 22:5n-3 to 22:6n-3.

Overall, our results argue against the direct conversion of 20:5n-3 to 22:6n-3, but are rather in favor of 22:6n-3 to 20:5n-3. Retroconversion of 22:6n-3 to 20:5n-3 has been reported in rat hepatocytes (39–41), rats (44,45), and humans (46,47). As 22:5n-3 is truly retroconverted to 20:5n-3, it will be difficult to study the unique effects of 22:6n-3 in rats as well as in humans as some of the 22:6n-3 will continuously be converted to 20:5n-3, and effects of 20:5n-3 may confound the search for the metabolic role of 22:6n-3, i.e., the secretion of VLDL triacylglycerol.

The present study reports that 20:5n-3, and not 22:6n-3, is the fatty acid primarily responsible for the triacylglycerol lowering effect of fish oil (Table 2). These effects are consistent with previous reports (15,44,48,49). Moreover, studies have shown that 20:5n-3 inhibits the triacylglycerol synthesis/secretion in rat (14,50), rabbit hepatocytes (51), HepG2 cells (11,52), and in perfused rat livers (10,24). Interestingly, at the second international congress of the International Society for the Study of Fatty Acids and Lipids (ISSFAL), held June 7–10, 1995, in Bethesda, Maryland, it was reported that 20:5n-3, and not 22:6n-3, appeared to be primarily responsible for the triacylglycerol lowering effect of fish oil in humans (living in Kansas City) (53). However, 22:6n-3 has also been reported to lower plasma triacylglycerol level in rats (45) and to lower triacylglycerol secretion from rat hepatocytes (3,54,55) in HepG2 cells (52) and perfused livers (10,12). These studies are relatively uniform in showing that 22:6n-3 as a substrate causes the cell to secrete reduced amounts of triacylglycerol. At the recent ISSFAL meeting (Grimsgaard, S., Bønaa, K.H., Hansen, J.B., Nordøy, A., and Ingelbretsen, O.C., unpublished data), it was reported that in the Tromsø study (Norway), 22:6n-3 was more efficient than 20:5n-3 in lowering plasma triacylglycerol concentration in humans. Moreover, in 1959 Worne and Smith (56) showed that 22:6n-3 reduced total serum lipids (triacylglycerol levels were not measured). In a more recent study (uncontrolled) by Hirai *et al.* (57), serum triacylglycerol levels were reduced after 22:6n-3 supplementation in Japanese Type IIb hyperlipidemia patients. These findings are in contrast to the human study reported at the 1995 ISSFAL meeting, intact animal data (15,22,44,48) (Table 2), and data from the perfused rat liver (24). The reason for this may be due to a higher basal level of 20:5n-3 and 22:6n-3 in humans from Norway and Japan compared to the people in Kansas City, as both Norwegians and Japanese probably consume more fish than the people living

TABLE 7
Changes in n-3 and n-6 Fatty Acids in Plasma Different Tissues of Rats Treated with EPA–EE, DHA–EE, Palmitic Acid (palm), and Corn Oil (corn) (control) for Three Months[a]

Fatty acid	Treatment	Fatty acid composition (mol%)				
		Plasma	Liver	Heart	Kidney	Epididymal fat
18:2n-6	Corn	26.1 ± 2.2	17.2 ± 0.8	25.3 ± 1.6	17.6 ± 1.7	33.1 ± 2.0
	EPA–EE	23.1 ± 2.0	18.2 ± 1.1	26.2 ± 2.4	17.6 ± 2.3	26.0 ± 2.4[b]
	DHA–EE	23.3 ± 1.5	16.3 ± 1.4	19.5 ± 1.8[b]	17.6 ± 1.3	25.9 ± 2.3[b]
	Palm	21.8 ± 4.4	18.2 ± 1.3	21.7 ± 1.7[b]	16.6 ± 1.9	24.6 ± 2.6[b]
18:3n-3	Corn	1.2 ± 0.1	0.3 ± 0.0	0.2 ± 0.2	0.4 ± 0.1	1.7 ± 0.2
	EPA–EE	1.2 ± 0.1	0.5 ± 0.1	0.2 ± 0.2	1.9 ± 0.2[b]	1.9 ± 0.2
	DHA–EE	1.4 ± 0.2	0.4 ± 0.1	0.3 ± 0.3	0.4 ± 0.2	2.0 ± 0.1
	Palm	0.9 ± 0.3	0.4 ± 0.2	0.3 ± 0.2	0.4 ± 0.1	1.7 ± 0.2
20:4n–3	Corn	trace	trace	trace	trace	trace
	EPA–EE	0.1 ± 0.0	trace	trace	0.1 ± 0.1	0.2 ± 0.1
	DHA–EE	0.1 ± 0.0	trace	trace	0.1 ± 0.0	0.2 ± 0.0
	Palm	trace	trace	trace	trace	trace
20:4n-6	Corn	13.8 ± 1.6	19.1 ± 1.1	15.3 ± 2.8	19.6 ± 1.2	0.6 ± 0.1
	EPA–EE	4.4 ± 0.6[b]	7.3 ± 0.9[b]	8.4 ± 0.8[b]	8.0 ± 1.6[b]	0.5 ± 0.2
	DHA–EE	5.7 ± 1.1[b]	8.8 ± 1.1[b]	6.8 ± 3.7[b]	12.2 ± 2.7[b]	0.5 ± 0.1
	Palm	11.1 ± 2.3	17.3 ± 2.9	15.7 ± 0.9	18.1 ± 3.2	0.4 ± 0.1
20:5n-3	Corn	0.9 ± 0.1	0.5 ± 0.1	0.1 ± 0.0	0.5 ± 0.3	0.1 ± 0.0
	EPA–EE	9.8 ± 3.7[b]	8.7 ± 1.7[b]	3.6 ± 0.5[b]	5.6 ± 1.3[b]	4.1 ± 1.4[b]
	DHA–EE	4.4 ± 0.9b	4.0 ± 0.9[b]	0.6 ± 0.2[b]	3.4 ± 0.5[b]	0.7 ± 0.1[b]
	Palm	0.9 ± 0.3	0.8 ± 0.2	0.2 ± 0.0	0.4 ± 0.2	0.1 ± 0.1
22:5n-3	Corn	0.9 ± 0.2	1.3 ± 0.3	1.4 ± 0.2	0.3 ± 0.1	0.3 ± 0.3
	EPA–EE	4.5 ± 0.8[b]	6.9 ± 0.9[b]	6.1 ± 0.5[b]	1.6 ± 0.2[b]	2.5 ± 0.5[b]
	DHA–EE	1.9 ± 0.5	1.9 ± 0.2	1.0 ± 0.3	0.6 ± 0.1[b]	1.0 ± 0.2[b]
	Palm	1.1 ± 0.3	1.4 ± 0.3	1.1 ± 0.2	0.3 ± 0.0	0.2 ± 0.1
22:6n-3	Corn	2.9 ± 0.5	5.7 ± 0.5	9.5 ± 2.7	1.6 ± 0.1	0.6 ± 0.2
	EPA–EE	3.0 ± 0.4	5.5 ± 1.0	8.0 ± 1.2	1.3 ± 0.1	1.4 ± 0.3[b]
	DHA–EE	10.7 ± 1.9[b]	14.8 ± 3.0[b]	14.8 ± 1.2[b]	4.5 ± 1.0[b]	6.8 ± 1.7[b]
	Palm	2.7 ± 1.0	6.7 ± 0.3	9.9 ± 3.4	1.8 ± 0.3	0.6 ± 0.3
n-3/n-6	Corn	0.14 ± 0.02	0.21 ± 0.02	0.31 ± 0.18	0.07 ± 0.01	0.08 ± 0.01
	EPA–EE	0.66 ± 0.12[b]	0.81 ± 0.11[b]	0.51 ± 0.10[b]	0.35 ± 0.05[b]	0.38 ± 0.06[b]
	DHA–EE	0.63 ± 0.11[b]	0.81 ± 0.18[b]	0.61 ± 0.21[b]	0.29 ± 0.07[b]	0.40 ± 0.05[b]
	Palm	0.16 ± 0.04	0.26 ± 0.03	0.35 ± 0.14	0.08 ± 0.01	0.11 ± 0.03

[a]Values are expressed as means ± SD. Abbreviations as in Table 1.
[b]Significantly different from the control at $P < 0.05$.

in Kansas City. The basal levels of 20:5n-3 and 22:6n-3 in our rats are also low. Whether the triacylglycerol-lowering effect by 22:6n-3 in human subjects from Norway and Japan could be ascribed to a conversion of 22:6n-3 to 20:5n-3, thereby increasing the level of 20:5n-3 (basal + retroconverted) should be considered. Jensen *et al.* (58), in an uncontrolled study, found no triacylglycerol-lowering effect with low doses of n-3, but did find a definite effect with a higher dose, confirming previous findings (59,60). Moreover, it has been shown that the more marked the triacylglycerol concentration at baseline, the greater its reduction by n-3 fatty acids (2,58,62–67), which may explain the striking response in hypertriacylglycerolemic patients (57,61,64).

In plasma the relative amount of 20:5n-3 and 22:6n-3 was almost identical (Table 7), indicating that the two n-3 fatty acids were equally well absorbed. However, in the liver and heart, the level of 20:5n-3 was less than half of the amount of 22:6n-3 (Table 7). The reason for this discrepancy could be ascribed to an increased β-oxidation of 20:5n-3 (68). Determining the effects of dietary 20:5n-3 and 22:6n-3 on hepatic fatty acid β-oxidation seems to be important to clarify the precise mechanism(s). Indeed, 20:5n-3 is reported to increase both the mitochondrial and peroxisomal β-oxidation (15,17–19,23), whereas 22:6n-3 increases peroxisomal β-oxidation (22).

An important finding in the present study was the observed decrease in plasma cholesterol level after prolonged EPA–EE and DHA–EE treatment (Table 2), and this is in agreement with previous reports (45,48,56,57). In perfused rat livers it has been shown that 22:6n-3 reduced the secretion of VLDL–cholesteryl esters similar to that with 20:5n-3, whereas only 20:5n-3 reduced the secretion of VLDL triacylglycerol (24). This study is in agreement with our plasma lipid data (Table 2).

Finally, in view of the cholesterol-lowering effect observed, we were able to explore how EPA–EE and DHA–EE treatments affected the enzymes in cholesterol and cholesterol–ester synthesis (HMG–CoA reductase and ACAT) and the transcription of the LDL-receptor and the LRP-4. HMG–CoA reductase is located mainly in endoplasmic reticulum, but the identification of the enzyme in peroxisomes, as

well as peroxisomal synthesis of dolichol and cholesterol, strongly supports the involvement of these organelles in intracellular lipid metabolism (39–41,65–67). It is well established that HMG–CoA reductase is rate-limiting in the synthesis of cholesterol under almost all experimental conditions (30,69). Prolonged administration of DHA–EE decreased the activity of microsomal HMG–CoA reductase, but had no effect on ACAT activity (Table 4). It is tempting to suggest that the observed hypocholesterolemic effect could be due to an inhibition of cholesterol biosynthesis.

LDL-receptor synthesis was probably unaffected after DHA–EE treatment to rats for three months as the LDL-receptor mRNA level was unchanged. Whether LDL-receptor activity was higher under the experimental conditions has not been addressed in this study. In a recent study by Spady (45), he found an induction in the LDL-receptor activity after both 20:5n-3 and 22:6n-3 supplementation, which is in contrast to a previous study by Roach *et al.* (70).

The underlying mechanisms for the observed decrease in plasma cholesterol are far from clear. Certain peroxisomal lipid metabolites have been implicated as inhibitors of sterol synthesis in cell cultures (71). In this study, peroxisomes seem to play a minor role in cholesterol biosynthesis during n-3 fatty acid treatments as peroxisomal HMG–CoA reductase activity remained unchanged (Table 4).

Prolonged feeding with pure EPA–EE, however, elevated the activities of ACAT and HMG–CoA reductase (Table 4) and raised the hepatic concentration of cholesterol (Table 3). This may be due to a compensatory effect in this long-term study, as the activities of these enzymes are suppressed during short-term EPA–EE treatment (Willumsen, N., Vaagenes, H., Asiedu, D.K., Lie, Ø., Rustan, A.C., and Berge, R.K., unpublished data). Because EPA–EE treatment lowered the plasma triacylglycerol (Table 2) and VLDL–triacylglycerol (data not shown) levels, the effect on cholesterol may be secondary to retarded triacylglycerol production. As VLDL is a precursor to LDL, it is conceivable that a reduction of VLDL secretion and of plasma VLDL levels will affect LDL formation, which might contribute to the cholesterol-lowering effect by EPA–EE treatment.

In summary, the present study suggests that 20:5n-3 and 22:6n-3 have different effects on hepatic synthesis and release of triacylglycerol and cholesterol and on some rate-limiting enzymes involved in these processes. EPA–EE feeding did not increase the amount of 22:6n-3, whereas DHA–EE increased the amount of 20:5n-3. The present study reports that 20:5n-3, and not 22:6n-3, is the fatty acid primarily responsible for the triacylglycerol-lowering effect of fish oil. Thus, this implies that it will be difficult to study the unique effects of 22:6n-3 as some of it will always be converted to 20:5n-3. Whether the effects were due to the 20:5n-3 generated from 22:6n-3, and not by 22:6n-3, must therefore be considered (41–50). The cholesterol-lowering effect after prolonged treatment with DHA–EE may, in part, be mediated by suppression of microsomal HMG–CoA reductase activity.

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Effects of Clofibrate on Lipids and Fatty Acids of Mouse Liver

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ABSTRACT: Clofibrate administration significantly altered the amount and fatty acid composition of lipids in mouse liver. The net content of phospholipids (PL) increased and that of triacylglycerols (TG) decreased concomitantly with liver enlargement in mice treated for two weeks with this drug (0.5% w/w in the food). The highest increase among PL was in phosphatidylcholine; other components either showed lower increases or, as in the case of sphingomyelin and the plasmalogens, decreased. In all lipid classes the treatment resulted in altered ratios between major saturates, between saturates and monoenes, and between major polyenes. Among these, 20:3n-6 and 22:5n-3 increased several-fold, and the 20:3n-6/20:4n-6 and 22:5n-3/22:6n-3 ratios increased due to a more active formation of the precursors than of the corresponding products. This change affected all glycerolipid classes. Liver sphingomyelin showed a relative enrichment in monoenoic fatty acids like 22:1 and 24:1, caused by a net decrease in the amount of saturates, particularly 22:0 and 24:0. The stimulated membrane proliferation imposed by clofibrate must increase phospholipid synthesis and, hence, the need for fatty acids. The results suggest that these demands are met mostly by TG acyl groups, either directly or after oxidation/desaturation processes. This was apparently the case for the polyenoic fatty acids of the n-6 and n-3 series. The longer chain (C_{22} and C_{24}) components decreased, suggesting that their oxidation was stimulated to provide part of the required (C_{20} and C_{22}) polyenes.
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Hypolipidemic drugs are used in the treatment of human hyperlipoproteinemias that are resistant to other therapeutic measures, such as diet manipulation, weight reduction, and exercise. The mechanism by which these drugs tend to lower elevated serum triglyceride and cholesterol levels has been an active research subject for years. Typical among these agents have been drugs belonging to the group of α-aryloxyisobutyric acids, such as clofibrate (1). This and other hypolipidemic chemicals have multiple actions at different loci in intact animals, their therapeutic use in humans being associated with many adverse reactions and undesirable effects.

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Abbreviations: DPG, diphosphatidylglycerol; GLC, gas–liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids; PUFA, polyunsaturated fatty acids; SM, sphingomyelin; TG, triacylglycerols.

In rodents, pharmacological doses of drugs like clofibrate and several structurally unrelated substances like herbicides and plasticizers are known to cause liver peroxisomes to proliferate in hepatic parenchymal cells (2). These "peroxisomal proliferators" cause induction of major peroxisomal enzymes, such as the ones that catalyze the β-oxidation of long- and very long chain fatty acids (3,4). Many studies on the physiological role of peroxisomes, their enzymes, and the molecular mechanisms of their induction have taken advantage of the use of peroxisomal proliferators as experimental tools to increase the yield of these organelles from rodent liver. The peroxisomal effect is but one of the changes effected by these chemicals in hepatocytes, however, as they also cause mitochondrial (5,6) and endoplasmic reticulum (1,2) proliferation and the activation of many enzymes known to be involved in the biosynthesis of lipids (see Ref. 7 and references therein). A nuclear protein receptor which is activated by peroxisomal proliferators, likely to mediate proliferator-induced gene expression, has been cloned from mouse (8) and rat (9), although its natural ligand has yet to be identified. In this study we have focused our interest on lipids, paying special attention to long- and very long chain polyunsaturated fatty acid (PUFA) components. We show that the administration of clofibric acid to mice under these conditions to induce peroxisome proliferation in rodents (10) leads to important modifications in the levels of major hepatic lipids, such as phosphatidylcholine (PC) and triacylglycerols (TG), affects the equilibrium between long- and very long chain (C_{24}) fatty acids in specific lipid classes like sphingomyelin (SM), and alters the proportions of relevant PUFA of the n-6 and n-3 series such as arachidonic (20:4n-6) and docosahexaenoic (22:6n-3) acids.

MATERIALS AND METHODS

Male albino mice of the BALB-C strain were used. Clofibric acid (Sigma Chemical Co., St. Louis, MO) was dissolved in acetone, food pellets (Nutrimentos, S.A., Buenos Aires, Argentina) were immersed in this solution for some time (0.5 g clofibric acid per 100 g pellets), and then the solvent was completely evaporated. Food from the same batch, used as control, was treated identically, except that it lacked the chemical. Control and experimental mice were allowed free access to

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water and food for two weeks, after which the animals were killed by decapitation and their livers removed and weighed. The total body weight did not change significantly after the treatment (28.0 ± 1.0 and 27.6 ± 2.0 g in control and experimental animals, respectively). Lipid extracts were prepared and washed (11), and aliquots were taken for the determination of lipid phosphorus. Total phospholipids (PL) and TG were separated by thin-layer chromatography (TLC) on silica gel G using hexane/ether (80:20, vol/vol). For analytical and preparative purposes, the PL were resolved into classes using two-dimensional TLC. Solvents and procedures described by Rouser *et al.* (12) were used for PL analysis. The choline and ethanolamine glycerophospholipids were eluted from the silica (13), the solvents evaporated, and the samples subjected to a brief treatment with HCl in chloroform/methanol to attack the alk-1-enyl bond in plasmenylcholine and plasmenylethanolamine (14). After this treatment, the samples were subjected to further TLC to separate the diacylphospholipids, PC, and phosphatidylethanolamine from the corresponding, plasmalogen-derived, lysoPC and lysophosphatidylethanolamine. After TLC, all lipids were treated with BF_3 in methanol (15) to obtain the fatty acid methyl esters, which were analyzed by gas–liquid chromatography (GLC). Methyl esters were also subjected to argentation TLC in order to separate them according to unsaturation. This was performed using 20% $AgNO_3$ in silica gel G as adsorbent and chloroform/methanol (95:5, vol/vol) as solvent. This preseparation procedure facilitated the detection of PUFA that are minor components of mouse liver and circumvented the common problem of partial co-elution of some PUFA with other fatty acids from packed GLC columns, especially when analyzing total PL or total lipids. For GLC, N_2 at 30 mL/min as carrier gas and two 2 m × 2 mm glass columns packed with a polar stationary phase (15% SP2330 on Chromosorb WAW 100/120; Supelco Inc., Bellefonte, PA) were used. The columns were connected to flame-ionization detectors operated in the dual-differential mode. The column oven temperature was programmed at 5°C/min from an initial temperature of 160°C to a final one of 220°C. Injector and detector temperatures were 220 and 230°C, respectively. Peak quantitation was performed by electronic integration. The results presented in the tables and in the text are mean values ± standard deviations. Statistical analyses were carried out using the two-tailed Student's *t*-test. *P* values lower than 0.05 were considered significant.

RESULTS

Treatment of mice with clofibric acid resulted in a significant increase in the amount of phosphorus recovered in lipid extracts from liver (23.4 ± 5.5 and 36.4 ± 4.4 μmol/liver in controls and in treated animals, respectively). Because hepatic weight also increased (1.18 ± 0.11 to 1.64 ± 0.04 g, i.e., about 39%), the amount of lipid phosphorus per gram of tissue did not change significantly (20.9 ± 2.9 and 22.2 ± 2.4 μmol/g, respectively). The synthesis of the major phospholipid classes was stimulated at approximately similar rates in response to the growth demands imposed by the proliferator, as suggested by their composition (Table 1), which was not significantly modified. The separation into subclasses, however, showed that PC underwent the greatest increase, as it was the major component of a growing mass of PL. Some glycerophospholipids increased at slower rates than PC, and still others decreased, as in the case of the plasmalogens and SM.

In contrast to the abovementioned increase in PL, the content per gram of tissue of liver TG decreased as a consequence of clofibric acid administration (Table 2). The fall in TG was conspicuously greater than the net PL gain. On a μg/g basis, the acyl groups of TG contributed to the total lipid acyl groups of liver with 52.8 ± 1.9% and 34.6 ± 2.4% in control and clofibric acid-treated mice, respectively. The TG/PL mole ratio decreased from 0.738 ± 0.053 in controls to 0.378 ± 0.050 in drug-treated animals.

The fatty acid composition of total lipid, PL, and TG (Table 2) shows that clofibrate administration gave rise to altered proportions of relevant saturated acids, monoenoic acids, and PUFA. The most marked change occurred in TG, where the proportion of most polyenoic fatty acids decreased significantly. Because TG levels were substantially reduced by the drug treatment (Table 2), the PUFA components of TG decreased to larger extents than other acyl groups. This was the case with 18:2n-6, 18:3n-6, and 18:3n-3, as well as major products of their elongation and desaturation, namely 20:4n-6, 22:5n-6, and 22:6n-3. In PL, in TG, and in the total lipid of liver, the drug induced significant increases in the 18:1/18:0 ratio and decreases in the 18:3n-6/18:2n-6, 20:4n-6/20:3n6, 22:5n-6/22:4n-6, and 22:6n-3/22:5n-3 ratios.

The fatty acid composition of representative glycerophospholipid classes (Table 3) shows that the proportions of saturated, monoenoic, and polyenoic fatty acids were altered in membrane lipids. All of them showed significantly increased percentages of 18:1, 20:3n-6, and 22:5n-3, in-

TABLE 1
Phospholipids of Mouse Liver[a]

	% of Total		μmol/g Liver	
Clofibrate	−	+	−	+
Phospholipid				
PC	43.2 ± 1.0	49.2 ± 0.7	280.2	338.6
CP	11.0 ± 1.0	5.1 ± 0.4[b]	71.3	35.1[b]
Total CGP	54.2 ± 0.7	54.3 ± 0.7	351.6	373.7
PE	26.3 ± 0.3	29.3 ± 0.4	170.6	201.6
EP	4.3 ± 0.3	2.0 ± 0.4[b]	27.9	13.8[b]
Total EGP	30.6 ± 0.5	31.3 ± 0.6	198.5	215.4
Phosphatidylinositol	5.8 ± 0.2	6.6 ± 0.6	37.6	45.4
Phosphatidylserine	2.6 ± 0.2	2.4 ± 0.4	16.9	16.5
Diphosphatidylglycerol	3.0 ± 0.1	2.8 ± 0.1	19.5	19.3
Sphingomyelin	3.8 ± 0.3	2.6 ± 0.4[b]	24.6	17.9[b]

[a]Values are means ± SD (n = 3–4 animals per group). Plus and minus signs indicate the presence or absence of clofibrate.
[b]Significant differences from control values, determined using the Student's *t*-test ($P < 0.05$); PC, phosphatidylcholine; CGP, choline glycerophospholipids; CP, plasmenylcholine; PE, phosphatidylethanolamine; EGP, ethanolamine glycerophospholipids; EP, plasmenylethanolamine. CGP values are totals of PC + CP values; EGP values are totals of PE + EP values.

TABLE 2
Effects of Clofibrate on the Fatty Acids of Mouse Liver[a]

	Total lipid		Phospholipid		Triacylglycerol	
Clofibrate	–	+	–	+	–	+
14:0	0.3 ± 0.02	0.2 ± 0.02	0.1 ± 0.01	—	0.4 ± 0.04	0.3 ± 0.04
15:0	0.2 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.2 ± 0.01	0.1 ± 0.01
16:0	20.1 ± 0.20	23.8 ± 0.49	22.3 ± 0.43	24.8 ± 0.25	18.1 ± 0.84	22.0 ± 0.31
16:1	1.6 ± 0.10	2.3 ± 0.15[b]	0.8 ± 0.05	1.2 ± 0.04[b]	2.3 ± 0.15	4.3 ± 0.18
17:0	0.4 ± 0.04	0.4 ± 0.02	0.6 ± 0.03	0.4 ± 0.03	0.3 ± 0.05	0.2 ± 0.02
17:1	0.2 ± 0.03	0.2 ± 0.02	0.2 ± 0.01	0.2 ± 0.02	0.3 ± 0.04	0.4 ± 0.01[b]
18:0	7.8 ± 0.32	7.6 ± 0.30	14.7 ± 0.33	11.2 ± 0.75	1.5 ± 0.39	1.3 ± 0.13
18:1	12.7 ± 0.93	16.3 ± 0.58[b]	6.8 ± 0.15	9.9 ± 0.23[b]	18.0 ± 1.85	27.6 ± 0.20[b]
18:2n-6	32.3 ± 0.52	22.8 ± 0.39[b]	18.4 ± 1.59	16.4 ± 0.30	44.8 ± 2.82	34.1 ± 0.22[b]
18:3n-6	1.1 ± 0.05	0.5 ± 0.02[b]	0.7 ± 0.03	0.4 ± 0.02	1.5 ± 0.15	0.8 ± 0.06[b]
18:3n-3	1.7 ± 0.05	0.8 ± 0.11[b]	0.2 ± 0.03	0.3 ± 0.03	3.0 ± 0.12	1.6 ± 0.30[b]
20:2	0.3 ± 0.01	0.2 ± 0.02	0.2 ± 0.04	0.2 ± 0.02	0.3 ± 0.01	0.5 ± 0.28
20:3n-9	0.3 ± 0.06	0.3 ± 0.05	0.3 ± 0.04	0.3 ± 0.02	0.2 ± 0.01	0.1 ± 0.03
20:3n-6	0.7 ± 0.05	2.7 ± 0.22[b]	0.9 ± 0.01	3.6 ± 0.17[b]	0.5 ± 0.06	1.2 ± 0.07[b]
20:4n-6	9.6 ± 0.42	11.9 ± 0.42[b]	17.1 ± 0.24	17.3 ± 0.39	2.8 ± 0.20	2.2 ± 0.13
22:4n-6	0.3 ± 0.05	0.3 ± 0.02	0.3 ± 0.03	0.3 ± 0.02	0.4 ± 0.08	0.4 ± 0.03
22:5n-6	0.2 ± 0.04	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.2 ± 0.06	0.1 ± 0.01
20:5n-3	0.3 ± 0.02	0.3 ± 0.02	0.2 ± 0.02	0.3 ± 0.02	0.4 ± 0.02	0.3 ± 0.04
22:5n-3	0.6 ± 0.05	1.0 ± 0.05[b]	0.7 ± 0.01	1.1 ± 0.04[b]	0.6 ± 0.03	0.8 ± 0.02[b]
22:6n-3	9.0 ± 0.58	7.9 ± 0.70	15.1 ± 0.20	11.6 ± 0.36[b]	3.5 ± 0.17	1.4 ± 0.07[b]
Others	0.3	0.3	0.2	0.3	0.7	0.3
mg/g tissue[c]	26.4 ± 1.90	20.9 ± 3.00	12.5 ± 2.40	13.3 ± 3.10	13.9 ± 1.20	7.6 ± 0.60

[a]Values are weight percent, as mean values ± SD (n = 3–4 samples).
[b]Significant differences from control values, determined using the Student's *t*-test ($P < 0.05$).
[c]Quantitated as the sum of fatty acid methyl esters.

creased 18:1/18:0 ratios, and decreased 20:4n-6/20:3n-6 and 22:6n-3/22:5n-3 ratios. Taking into account the compositional changes (Tables 2 and 3) and the alterations in lipid amounts (Tables 1 and 2), the greatest rises in 18:1, 20:3n-6, and 22:5n-3 occurred in PC, and the greatest decreases in 20:4n-6 and 22:6n-3 took place in TG.

The increase induced by clofibrate in 18:1 in all PL classes (Table 3) was mirrored by a concomitant relative decrease in

TABLE 3
Effects of Clofibrate on Mouse Liver Glycerophospholipid Fatty Acids[a]

	PC		PE		PI		PS		DPG	
Clofibrate	–	+	–	+	–	+	–	+	–	+
14:0	0.2	0.1	—	—	—	—	—	—	0.1	0.2
15:0	0.2	0.1	—	—	—	—	—	—	—	0.1
16:0	32.4	34.4	20.5	18.9	3.4	5.2	0.8	1.1	2.8	4.4
16:1	1.1	1.3	0.5	0.8	—	—	—	—	1.2	4.3[b]
17:0	0.6	0.4	0.6	0.5	0.3	0.4	0.2	0.2	—	—
17:1	0.2	0.2	0.1	0.2	—	—	0.1	0.2	—	0.2
18:0	10.5	6.6[b]	18.5	14.9[b]	43.2	39.9[b]	36.4	32.2[b]	1.1	1.7[b]
18:1n-9	6.7	9.3[b]	9.3	13.1[b]	2.7	4.4[b]	2.6	3.1[b]	11.3	21.1[b]
18:2n-6	23.8	22.2	8.9	6.9	3.1	2.6	0.4	0.4	74.5	60.0[b]
18:3n-6	0.5	0.3	—	—	0.1	0.2	1.4	1.1	0.4	0.2
18:3n-3	0.2	—	0.2	0.1	—	—	0.4	0.2	—	—
20:2	0.2	0.2	0.3	0.3	—	0.1	0.2	0.2	0.3	0.2
20:3n-9	0.2	0.3	0.2	0.2	—	0.3	0.2	0.1	2.5	0.6[b]
20:3n-6	0.8	4.4[b]	0.6	1.6[b]	1.1	4.2[b]	1.2	2.1[b]	0.7	2.7[b]
20:4n-6	14.1	12.4	15.5	20.8	44.1	40.2	18.1	22.8	1.6	1.6
22:4n-6	0.1	0.1	0.2	0.2	0.2	0.3	1.5	1.5	0.1	0.1
22:5n-6	0.1	0.1	0.2	0.2	0.5	0.5	1.1	0.7	0.4	0.3
20:5n-3	0.2	0.2	0.2	0.3	0.7	0.7	0.4	0.9	0.2	0.2
22:5n-3	0.3	0.7[b]	0.8	1.5[b]	0.1	0.3[b]	1.7	2.1[b]	—	0.2[b]
22:6n-3	7.6	6.7	23.4	19.5[b]	0.5	0.7	33.3	31.1	2.8	1.9[b]

[a]Values are weight percent, as mean values ± SD (n = 3–4 samples). PC, Phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; DPG, diphosphatidylglycerol.
[b]Significant differences from control values, determined using the Student's *t*-test ($P < 0.05$).

TABLE 4
Fatty Acids of Mouse Liver Sphingomyelin[a]

Clofibrate	–	+
14:0	0.2 ± 0.04	0.3 ± 0.02
14:1	0.2 ± 0.01	0.2 ± 0.02
15:0	0.1	trace[b]
15:1	0.1	0.1
16:0 (+16:1)	11.4 ± 1.00	10.4 ± 0.50
17:0	0.3 ± 0.04	0.2 ± 0.02
18:0	5.4 ± 0.33	3.7 ± 0.21[c]
18:1	2.2 ± 0.14	2.1 ± 0.50
19:0	1.2 ± 0.05	0.7 ± 0.04
20:0	2.7 ± 0.08	2.2 ± 0.25[c]
21:0	0.5 ± 0.04	0.4 ± 0.04
21:1	0.1 ± 0.01	trace
22:0	26.3 ± 1.64	18.4 ± 0.25[c]
22:1	2.0 ± 0.07	3.3 ± 0.23
23:0	9.2 ± 0.19	9.4 ± 0.76
23:1	1.4 ± 0.02	1.5 ± 0.03
24:0	14.3 ± 0.80	12.0 ± 0.79[c]
24:1	19.0 ± 0.42	30.6 ± 0.66[c]
25:0 (+24:2)	2.6 ± 0.20	3.2 ± 0.36[c]
25:1	0.8 ± 0.23	1.3 ± 0.18[c]

[a]Values are weight percent, as mean values ± SD (n = 3–4 samples).
[b]Trace amounts percentage lower than 0.05%.
[c]Significant differences from control values, determined using the Student's *t*-test ($P < 0.05$).

18:0 such that the sum 18:0 + 18:1 was little affected, whereas the 18:1/18:0 ratio increased in all cases after clofibrate. In diphosphatidylglycerol (DPG), a lipid naturally poor in 18:0 and rich in 18:1, drug treatment resulted in a marked increase in 18:1 as well as in other monoenes. This occurred in the presence of an almost parallel decrease in the percentage of the major DPG component, 18:2n-6. Like the other constituents, DPG showed a decrease in the 20:4n-6/20:3n-6 and 22:6n-3/22:5n-3 ratios. DPG was the lipid to most clearly show a considerably diminished level of 20:3n-9 and a decreased 20:3n-9/20:2n-9 ratio. This is consistent with the decrease observed in the 20:4n-6/20:3n-6 ratio, as in both pairs of fatty acids the components are related *via* a 5-desaturation. The ratio 22:5n-6/22:4n-6 decreased in DPG as in most lipids, also in line with the decreases seen for 22:6n-3/22:5n-3, as the components of both pairs are related by a 4-desaturation.

SM differs from the glycerophospholipids in that it contains almost exclusively saturated and monoenoic fatty acids, being rich in very long chain components (Table 4). After clofibrate treatment, the percentage of all saturates of SM decreased (from 74.2 to 61.0%), whereas that of monoenes increased (from 25.8 to 39.0%), the saturate/monoene ratio changing from 2.87 to 1.56. The major saturated components of SM, 22:0 and 24:0, decreased; the major monoenes, 22:1 and 24:1, increased. Because the concentration of SM in liver was reduced with the drug (Table 1), it is clear from the results in Table 4 that the monoenes were much less affected than were the saturates.

The percentages of n-6 and n-3 PUFA in total lipid, PL, and TG are shown in Table 5. The changes induced by clofibrate in each of these groups are more clearly apparent here than in Table 2, where PUFA are together and their proportions are relatively minimized by the contribution of major saturated and monoenoic fatty acids. In controls, nearly half the fatty acids of TG and ~40% of those of PL were n-6 PUFA, whereas close to 9% of the fatty acids of TG and 16% of those of PL were n-3 PUFA (Table 5). Of the n-6 PUFA, as much as 90% of those of TG was made up of 18:2n-6, and ~95% of those of PL was accounted for by 18:2 and 20:4n-6. Of the n-3 PUFA, as much as 80% of those of TG was accounted for by 18:3n-3 and 22:6n-3, and the bulk (>90%) of those of PL was 22:6n-3. In controls, TG had larger basal proportions (Table 5) and concentrations than PL of all n-6 PUFA other than 20:3n-6 and 20:4n-6, and also of all n-3 PUFA other than 22:6 n-3.

The sum of n-6 and that of n-3 PUFA in liver PL was maintained at similar percentages with respect to other fatty acids in controls and clofibrate-treated animals, whereas in TG the proportion of n-6 and n-3 PUFA, but especially the latter, decreased substantially in relation to other fatty acids (Table 5). Among n-6 PUFA, the most remarkable effect of clofibrate on the total lipid composition was a large increase in the percentage of 20:3n-6, the metabolic precursor of 20:4n-6, with a concomitant decrease in that of 18:2n-6, 18:3n-6, and 24:5n-6. Among n-3 PUFA, the drug induced a large increase in the percentage of 22:5n-3, the metabolic precursor of 22:6n-3, with a concomitant decrease in that of 18:3n-3, 18:4n-3, and 24:6n-3. Taking into account the total amounts and the percentages of n-6 and n-3 PUFA (Table 5), it results that the significant decreases observed in the percentages of liver lipid 18:2n-6 and 24:4n-6 among n-6 PUFA, and in those of 18:3n-3 and 24:6n-3 among n-3 PUFA, were mostly, if not exclusively, contributed by TG. Conversely, the significant increases observed in the percentages of liver lipid 20:3n-6 and 20:4n-6 among n-6 PUFA, and of 22:5n-3 and 22:6n-3 among n-3 PUFA, were virtually exclusively contributed by PL. With the exception of 20:3n-6, all PUFA decreased in TG under the effects of clofibrate, whereas the concentrations of the major PUFA components of PL, 18:2n-6, 20:4n-6, and 22:6n-3, were preserved close to those of control conditions.

DISCUSSION

The data presented here show that the administration of clofibric acid to mice resulted in profound qualitative and quantitative modifications of hepatic lipids and their fatty acids. The major lipid effects were an increase in the amount of phospholipids and a decrease in that of TG, changes which are consistent with the well-known effect of clofibrate as plasma triglyceride-lowering agent. The proliferation of hepatic peroxisomes and mitochondria concomitantly with enzymes (10,16) that participate in fatty acid β-oxidation in both organelles is another of the typical biochemical responses of rodents to the challenges imposed by clofibrate and pharmacologically-related chemicals. In addition, drugs effecting peroxisomal proliferation in relatively high doses are well-known inducers of liver hypertrophy and hyperplasia (17,18).

TABLE 5
Effects of Clofibrate on the Percentages of Polyenoic Fatty Acids of the n-6 and n-3 Series in Mouse Liver[a]

	Total lipid		Phospholipids		Triacylglycerols	
Clofibrate	−	+	−	+	−	+
18:2n-6	73.38 ± 0.46	59.72 ± 0.30[e]	49.04 ± 1.00	43.41 ± 0.47[e]	89.50 ± 0.44	88.40 ± 0.67
18:3n-6	2.20 ± 0.20	1.20 ± 0.02[e]	1.74 ± 0.10	0.97 ± 0.04[e]	2.26 ± 0.10	1.55 ± 0.11[e]
20:3n-6	1.60 ± 0.03	7.42 ± 0.16[e]	2.48 ± 0.06	9.49 ± 0.21[e]	0.96 ± 0.15	3.14 ± 0.15[e]
20:4n-6	21.44 ± 0.32	30.47 ± 0.48[e]	45.57 ± 1.35	44.92 ± 0.49	5.63 ± 0.51	5.63 ± 0.25
22:4n-6	0.88 ± 0.11	0.83 ± 0.15	0.79 ± 0.15	0.84 ± 0.00	1.06 ± 0.16	1.05 ± 0.05
24:4n-6	0.05 ± 0.02	0.05 ± 0.00	0.05 ± 0.00	0.15 ± 0.00[e]	0.04 ± 0.01	0.04 ± 0.01
22:5n-6	0.40 ± 0.03	0.19 ± 0.01[e]	0.32 ± 0.05	0.20 ± 0.03[e]	0.48 ± 0.11	0.18 ± 0.02[e]
24:5n-6	0.05 ± 0.00	0.02 ± 0.00[e]	0.01 ± 0.00	0.02 ± 0.01	0.08 ± 0.02	0.03 ± 0.01[e]
% of total fatty acids[b]	45.6 ± 1.0	37.3 ± 0.6[e]	38.7 ± 1.8	38.8 ± 1.0	50.9 ± 2.5	38.8 ± 0.1[e]
18:3n-3	14.60 ± 0.80	7.40 ± 0.35[e]	1.33 ± 0.06	2.09 ± 0.12[e]	38.57 ± 0.21	37.65 ± 4.02
18:4n-3	0.90 ± 0.00	0.60 ± 0.00	0.72 ± 0.00	0.59 ± 0.00	1.19 ± 0.20	0.63 ± 0.20
20:4n-3	0.59 ± 0.16	0.50 ± 0.08	0.66 ± 0.00	0.37 ± 0.02	0.74 ± 0.06	1.35 ± 0.41
20:5n-3	2.49 ± 0.11	2.18 ± 0.11	0.90 ± 0.07	1.41 ± 0.07[e]	5.38 ± 0.43	6.05 ± 0.91
22:5n-3	5.19 ± 0.10	10.12 ± 0.11[e]	4.06 ± 0.11	8.14 ± 0.41[e]	7.31 ± 0.54	21.76 ± 1.34[e]
24:5n-3	0.41 ± 0.01	0.24 ± 0.03[e]	0.12 ± 0.00	0.10 ± 0.00[e]	0.89 ± 0.13	0.94 ± 0.11
22:6n-3	74.83 ± 1.12	78.50 ± 1.11[e]	91.57 ± 0.11	86..76 ± 0.42[e]	44.40 ± 0.33	30.94 ± 1.74[e]
24:6n-3	0.95 ± 0.03	0.48 ± 0.02[e]	0.64 ± 0.02	0.43 ± 0.01[e]	1.48 ± 0.35	0.67 ± 0.12[e]
% of total fatty acids[b]	13.1 ± 1.0	12.0 ± 0.5	15.7 ± 1.1	13.4 ± 0.3	8.7 ± 1.0	4.1 ± 0.2[e]
n-6/n-3 ratio[c]	3.6 ± 0.2	3.4 ± 0.5	2.6 ± 0.3	2.9 ± 0.1	6.5 ± 0.2	9.5 ± 0.6[e]
Contents[d]						
n-6	11.11 ± 0.24	7.91 ± 0.13[e]	4.49 ± 0.21	5.14 ± 0.13	6.62 ± 0.33	2.76 ± 0.01[e]
n-3	3.02 ± 0.23	1.97 ± 0.08[e]	1.87 ± 0.13	1.70 ± 0.04	1.18 ± 0.14	0.27 ± 0.01[e]

[a]Fatty acid methyl esters were separated by means of argentation thin-layer chromatography to facilitate the detection of minor components. Values are percentages ± SD (n = 3–4 samples) of total n-6 (or n-3) polyunsaturated fatty acids (PUFA), calculated separately.
[b]Sum of all n-6 (or n-3) PUFA components in relation to the total fatty acids of each lipid (%).
[c]Ratios between the sums given in *b*.
[d]Amounts, in mg/g of tissue.
[e]Significant differences from control values, determined using the Student's *t*-test ($P < 0.05$).

An increased phospholipid biosynthesis to supply the cellular membranes required for this growth is consistent with the increase in liver weight and phospholipid mass (with virtually unchanged phospholipid levels per unit weight) observed here. Clofibrate-induced fatty acid oxidation, TG depletion, and PL formation are thus related. In rat liver (7) and kidney (19), it has been demonstrated that peroxisomal β-oxidation and microsomal 1-acylglycerophosphocholine acyltransferase are co-induced after administration of several peroxisomal proliferators. Other enzymes participating in hepatic lipid synthesis whose activity increase in response to these drugs are CTP:phosphocholine acyltransferase (20), acyl-CoA synthetase, 1-acylglycerophosphate acyltransferase (21), and fatty acid desaturases (22). The present results suggest that some of the fatty acids whose oxidation is stimulated by clofibrate in liver, from saturated to polyunsaturated, may originate in endogenous TG. The purpose of such fatty acid oxidation could be in part catabolic, as energy demands increase dramatically, and in part anabolic, as higher requirements for more fatty acids, including PUFA, are to be met. To sustain the stimulated synthesis of glycerophospholipids, remodelling of fatty acid pre-existing in the tissue could meet part of the requirements for important PUFA, such as 20:4n-6 and 22:6n-3.

The chain shortening of long-chain fatty acids which are poorly oxidized in mitochondria has been considered a physiologically relevant role of peroxisomal β-oxidation (23–25). In rodent liver, very long saturates like behenic, lignoceric, and cerotic acids, in the form of acyl-CoAs, are preferentially, if not exclusively, oxidized in peroxisomes (26–28), as mitochondria lack the necessary acyl-CoA (e.g., lignoceroyl CoA) ligase (29). The significant reduction of SM observed here, as it was concurrent with a decrease that selectively affected its very long saturated acyl chains, could be contingent upon stimulated oxidation of fatty acids in peroxisomes, this process consuming part of the substrates normally used for synthesizing SM (e.g., 24:0). Very long chain polyenoic fatty acids, such as those having 24 carbons, could also be chain-shortened in peroxisomes to give the major 20- and 22-carbon PUFA. Such oxidation, stimulated by peroxisomal proliferation, would explain the present observations of decreased levels of very long C_{24} PUFA in the liver of clofibric acid-treated mice. The novel observation we have made is that the TG account for most of this decrease.

In addition to directly affecting fatty acid oxidation, measured through $^{14}CO_2$ production, feeding clofibric acid and other structurally unrelated peroxisomal proliferators results in an accelerated conversion of [^{14}C]-labeled 18:2n-6, 18:3n-6, and 20:3n-6 to their desaturated and elongated metabolites, [^{14}C] 20:3n-6 and 20:4n-6 (22). This response was ascribed to induction of hepatic fatty acid desaturases, as it could be prevented by pretreatment of rats with cycloheximide. The increase observed here in 20:4n-6 content in liver PL agrees with these findings and interpretations. The disproportionate increase of 20:3n-6 with respect to 20:4n-6 suggests that: (i) the 20:4 newly formed was in part retroconverted, or (ii) even though stimulated, 5-desaturase synthesized less product than could have been made from the available substrate. The latter possibility is consistent with the fact that desaturases are rate-limiting steps in PUFA formation pathways (30). Our results show that under the influence of clofibrate, an analogous phenomenon affected the n-3 PUFA, as the proportion of 22:5n-3 increased more than that of the 4-desaturation product, 22:6n-3.

The observed accumulation in the total lipid of 20:3n-6 and 22:5n-3 with a concomitant net depletion of 18:2n-6 and 18:3n-3 on the one hand, and of 24:5n-6 and 24:6n-3 on the other hand, support the idea that elongation/desaturation of the 18 carbon precursors occurred at rates higher than normal in the presence of an accelerated chain-shortening of the longest n-6 and n-3 PUFA. Both mechanisms could contribute to meet the increased demands for the major 20:4n-6 and 22:6n-3 destined to phospholipids. The TG could be actively involved as donors of fatty acid precursors for both processes, as long-chain precursors (18 carbons) as well as very long chain products (24 carbons) decreased several-fold in TG under the effects of clofibrate without appearing as such in PL. The fact that in liver under basal conditions there were relatively larger amounts of most PUFA other than 20:4 and 22:6 in TG than in PL supports this possibility.

The changes induced by clofibric acid on mouse liver fatty acids agree with previous results in the rat (22) as far as n-6 PUFA are concerned. The corresponding PUFA of the n-3 series are shown here to be affected, even more drastically so than the n-6 polyenes. The relative effects on n-6 or n-3 PUFA could depend on the diet, in this case reflecting the relatively larger abundance of the former with respect to the latter in the standard rodent diet used, containing sunflower oil as the major source of fat (large n-6/n-3 ratio). Previous work has shown that the greater the proportion of n-3 PUFA in the diet, the lower the concentration of TG in plasma and the larger in liver (31). In rats fed a diet supplemented with a high amount of fish oil (31,32), or given separately the main constituents of fish oil, 20:5n-3 and 22:6n-3 (33), hepatic peroxisomal β-oxidation was stimulated. Because the effects of clofibrate on liver lipids, and consequently on blood lipids, could depend upon the type of polyenoic fatty acids (n-6, n-3, or n-9) fed to the animals, the effect of the simultaneous administration of hypolipidemic agents like clofibrate and hypolipidemic diets based on different types of dietary fatty acids deserves further investigation.

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Influence of Triacylglycerol Structure and Fatty Acid Profile of Dietary Fats on Milk Triacylglycerols in the Rat. A Two-Generation Study

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ABSTRACT: We investigated the influence of dietary fatty acid profile and triacylglycerol structure on the fatty acid profile and triacylglycerol structure of milk lipids in two generations of rats. Three groups of rats received diets containing 20% fat of which approximately 20% was n-3 fatty acids located in different positions of the triacylglycerol: a fish oil-based diet [docosahexaenoic acid (22:6n-3) predominantly in the *sn*-2 position], a seal oil-based diet (22:6n-3) predominantly in the *sn*-1/*sn*-3 position, or a plant oil-based diet [α-linolenic acid (18:3n-3) distributed evenly between the three positions]. This design allowed us to investigate (i) the effect of the triacylglycerol structure of the dietary fat; (ii) the effect of receiving the n-3 fatty acids as long-chain derivatives or as the precursor, 18:3n-3; and (iii) the long-term effects over two generations. The fatty acid profiles of the milk lipids largely reflected the diets, but in the second generation, the level of medium-chain fatty acids was higher ($P < 0.05$) in the milk from rats fed the fish oil diet (24%) compared with the other dietary groups (15 and 18%). This suggests an increased endogenous synthesis of fatty acids in the mammary glands of the fish oil-fed rats. The levels of long-chain n-3 fatty acids in milk were higher ($P < 0.05$) in rats fed marine oils (8–12%) compared with rats fed vegetable oil (1%) in both generations. The level of long-chain n-3 fatty acids was significantly higher in the milk from the fish oil-fed rats (12.3%) compared to the seal- oil fed rats (8.0%) in the first generation, but not in the second generation (8.9 vs. 9.1%). The general structure of milk triacylglycerols was maintained in the three experimental groups with 16:0 acylated in the *sn*-2 position and 18:1 in the *sn*-1/*sn*-3 positions. The triacylglycerol structure of mammalian milk appears to be conserved even during extreme dietary manipulation over two generations and an extensive enrichment with long-chain n-3 polyunsaturated fatty acids requires their presence in the diet.

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During the embryonic and early postnatal period, long-chain polyunsaturated fatty acids (PUFA), especially docosahexaenoic acid (22:6n-3), and arachidonic acid (20:4n-6), accumulate in the nervous system, including brain and retina. These fatty acids constitute up to 60% of the fatty acids in the photoreceptor outer segment membrane in the retina and in the synaptic membranes in the brain (1). The largest accretion of the PUFA in the nervous system takes place during the last trimester and the first 6–10 mon after birth in humans (2), and in the first 15 d after birth in the rat (3). The mammalian fetus receives the PUFA from the mother by placental transfer and the newborn mammal obtains them from the maternal milk.

We have demonstrated in a two-generation experiment that intake of fish oil, rich in eicosapentaenoic acid (20:5n-3) and 22:6n-3, compared to linseed oil, rich in 18:3n-3, is accompanied by elevated levels of 22:6n-3 in brain and heart phosphatidylethanolamine (4). We have also shown that the lymphatic absorption of a fatty acid is enhanced when this fatty acid is situated in the *sn*-2 position of dietary triacylglycerol (TAG) (5). About 75% of the long-chain PUFA in the *sn*-2 position in the dietary TAG are conserved during the absorption (6). Intravenously injected chylomicrons prepared from rats fed oils with different TAG structures are cleared at different rates (7). The TAG structure will therefore exert short-term effects on absorption, metabolism, and distribution of dietary fats in the organism. In the present long-term investigation, we examined whether the structure of maternal dietary TAG affects the structure and the fatty acid profiles of the milk TAG, with particular reference to n-3 PUFA. Differences in milk TAG could affect the absorption of fatty acids from the milk and the following tissue distribution in the suckling offspring.

MATERIALS AND METHODS

Animals and diets. The experiments were approved by the Danish Animal Experiments Inspectorate. Weanling female Wistar rats were purchased from Møllegård Breeding Centre A/S, Ll (Skensved, Denmark). They were housed in plastic cages and provided with tap water *ad libitum*. The temperature was maintained at 24°C, the relative humidity was 45%,

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Abbreviations: GLC, gas–liquid chromatography; MCFA, medium-chain fatty acids; PUFA, polyunsaturated fatty acids; TAG, triacylglycerol; TLC, thin-layer chromatography.

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and the light/dark cycle was 12 h/12 h. The rats were divided into three groups of four rats, and received *ad libitum* diets composed of (in wt%): 40% starch, 20% fat, 20% casein, 10% sucrose, 5% salt mixture, 4% cellulose powder, 0.5% choline chloride, and 0.5% vitamin mixture (8). New batches were prepared every third week and stored at −40°C. In the fish oil diet, the fat component was 60% fish oil (Okholm olie; Thyborøn Andelsfiskeindustri, Thybøron, Denmark), 24% corn oil (FDB, Albertslund, Denmark), and 16% olive oil (Irma A/S, Rødovre, Denmark). In the seal oil diet it was 60% seal oil (made from seal blubber from Royal Greenland A/S, Greenland), 26% corn oil, 6% palm oil (Palmotex, Aarhus Oliefabrik A/S, Aarhus, Denmark), 6% olive oil, and 2% linseed oil (Nomeco A/S, Copenhagen, Denmark). The dietary oil in the vegetable oil diet was made from: 42% olive oil, 29% linseed oil, 15% palm oil, 10% corn oil, and 4% grape kernel oil (Dansk Supermarked A/S, Lyngby, Denmark). By these combinations of the oils, the amounts of polyunsaturated, monounsaturated, and saturated fatty acids in the three diets were similar (Table 1). The fish and seal oil diets differed mainly in the TAG structure, but it was not possible to make the fatty acid profiles completely similar due to the content of docosapentaenoic acid (22:5n-3) in the seal oil. The total amounts of n-3 fatty acids were nevertheless almost similar in all three diets, in the vegetable oil diet being 18:3n-3, whereas in the other diets it was 20:5n-3, 22:5n-3, and 22:6n-3 (Table 1). After five weeks, the rats were mated with male rats receiving ordinary chow diet. After approximately 20 d of pregnancy, the pups were delivered. Three weeks later the pups were separated from their mothers, and a minimum of seven females selected at random from each group were used in the succeeding part of the study. At approximately 14 wk of age, they were mated with male rats receiving an ordinary chow diet, and went through the same procedure as the first generation.

Collection of milk. The pups were separated from the mothers. After about four hours, the mothers received 0.2 mL Oxytocine intraperitoneally (10 IE/mL; Leo Vet, Ballerup, Denmark), and were sedated with 0.2 mL/100 g body weight of a mixture of Hypnorm (10 mg/mL fluanisonum, 0.2 mg/mL fentsnylum; Janssen, Oxford, United Kingdom):water (redestilled), 1:1, and Dormicum (Roche, Basel, Switzerland; 5 mg/mL)/water (redistilled), 1:1. The papillae were cleaned with ethanol, and the rats were milked manually with a yield of 1–2 mL of milk per rat per session. This was performed weekly for three weeks. The milk samples were stored at −20°C until analysis.

Analysis of milk lipids. The milk samples were extracted by the method of Folch *et al.* (9). The total fatty acid composition in the milk was determined by methylation with KOH in methanol (10), and the fatty acid methyl esters were analyzed by gas–liquid chromatography (GLC), using a Fisons Instruments HRGC Mega 2 series 8560 gas chromatograph with flame-ionization detection (Fisons Instruments, Milano, Italy), and a BPX-70 fused silica capillary column (25 m × 0.22 mm i.d.) (SGE, Victoria, Australia). On-column injection was used. Initial oven temperature was 40°C for 2 min, followed by a temperature gradient of 10°C/min until 150°C, followed by a gradient of 3°C/min until 215°C, followed by a gradient of 5°C/min, until a final oven temperature of 220°C, which was held for 3 min. The fatty acids were identified by

TABLE 1
Fatty Acid Composition of the Dietary Fats[a]

	Seal oil diet		Fish oil diet		Vegetable oil diet	
Fatty acids	TAG	*sn*-2	TAG	*sn*-2	TAG	*sn*-2
MCFA[b]	0.1 ± 0.1	0.2 ± 0.0	0.1 ± 0.1	0.7 ± 0.1	—	—
14:0	3.3 ± 0.1	6.6 ± 0.1	2.5 ± 0.3	2.7 ± 0.3	—	—
16:0	11.8 ± 4.5	9.7 ± 0.3	14.1 ± 0.7	10.8 ± 0.5	12.8 ± 0.4	2.6 ± 0.4
16:1n-7	8.5 ± 1.5	17.2 ± 0.1	3.1 ± 0.6	3.0 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
18:0	2.2 ± 0.1	0.8 ± 0.2	2.7 ± 0.1	1.9 ± 2.2	3.1 ± 0.5	0.2 ± 0.0
18:1n-7/9	28.0 ± 0.8	33.8 ± 0.2	34.0 ± 0.2	27.4 ± 0.7	47.7 ± 0.2	56.2 ± 0.3
18:2n-6	16.8 ± 0.2	22.4 ± 0.5	16.4 ± 0.9	20.5 ± 1.0	19.7 ± 0.9	24.9 ± 0.3
18:3n-3	1.5 ± 0.7	2.0 ± 0.8	2.1 ± 0.0	1.8 ± 0.1	16.6 ± 0.2	15.9 ± 0.0
20:0	1.3 ± 0.3	0.6 ± 0.0	—	—	—	—
20:1	6.5 ± 0.2	1.4 ± 0.1	2.3 ± 0.2	0.4 ± 0.1	—	—
20:4n-6	0.1 ± 0.0	0.1 ± 0.0	—	—	—	—
20:5n-3	8.9 ± 0.5	0.8 ± 0.2	9.8 ± 0.2	7.7 ± 0.6	—	—
22:5n-3	2.7 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	1.1 ± 0.1	—	—
22:6n-3	7.5 ± 0.8	1.3 ± 0.4	10.3 ± 0.3	19.0 ± 1.1	—	—
Saturated	18.7	19.2	19.4	17.0	15.9	2.8
Monounsaturated	43.0	53.8	39.4	30.9	47.8	56.5
n-6 PUFA	16.9	22.5	17.7	22.4	19.7	24.9
n-3 PUFA	20.6	4.5	22.7	29.7	16.6	15.9

[a]Determined by gas–liquid chromatography (GLC) for triacylglycerols (TAG) or by regiospecific degradation followed by thin-layer chromatography and GLC as described in the text. Mean ± standard error of three determinations; PUFA, polyunsaturated fatty acids.
[b]Medium-chain fatty acids (MCFA) include $C_{6:0}$ to $C_{12:0}$.

comparison with authentic standards (Nu-Chek-Prep Inc., Elysian, MN).

Regiospecific analysis of TAG. Extracted lipid from milk samples or dietary oils were dissolved in diethyl ether in a 50-mL round-bottomed flask (11). While stirring with a magnetic stirrer, 0.2 mL allyl magnesium bromide was added, and the reaction was allowed to proceed for 60 s. Then 8 mL of acid buffer was added (0.27 M HCl in 0.4 M boric acid solution), both phases were transferred to a 30 mL test tube, and the water phase was removed. The ether phase was washed twice with 8-mL boric acid solution (0.4 M). The ether phase was dried with sodium sulphate, and the ether was evaporated under nitrogen. The glycerides were dissolved in 100 μL chloroform and separated into TAG, diacylglycerol, monoacylglycerols, and tertiary alcohol, using boric acid (0.4 M) impregnated thin-layer chromatography (TLC)-plates (E. Merck, Darmstadt, Germany) as described by Thomas *et al.* (12). The regiospecific analysis was based on the fatty acid profile of the *sn*-2 monoglyceride, in which the acyl migration of the fatty acids was <2% based on analysis of an actual standard (1,3-dioleoyl-2-palmitoyl-glycerol, 99%; Sigma, St. Louis, MO). The *sn*-2 monoglyceride band was scraped off the TLC-plate and extracted from the silica gel with 3 × 1 mL diethyl ether. The ether was evaporated under nitrogen, the glycerides dissolved in hexane, methylated, and analyzed by GLC as described above.

Statistics. All data were analyzed by analysis of variance. When significant differences were present, means were compared by Duncan's Multiple Range Test with a 5% level of significance.

RESULTS

The fatty acid profiles of the milk TAG were generally similar between the three dietary groups as well as first and second generation (Table 2). The medium-chain fatty acids, 6:0 to 14:0, were generally similar in the three groups of the first generation, but in the second generation significantly increased ($P < 0.05$) in the fish oil group and decreased ($P < 0.05$) in the vegetable oil group. The saturated fatty acids, 16:0 and 18:0, in the milk TAG did not appear to be affected by the dietary fat or the generations examined. The levels of the monounsaturated fatty acids showed diet differences. The major fatty acid in milk, 18:1n-9, was present at higher ($P < 0.05$) levels in milk from the vegetable oil-fed rats compared to the other groups and increased ($P < 0.05$) from first to second generation. The highest levels ($P < 0.05$) of palmitoleic acid (16:1n-7) and 20:1 were present in the seal oil group, where they appeared to accumulate, and the lowest ($P < 0.05$) levels were observed in the vegetable oil group. The concentration of the n-6 fatty acids, 18:2n-6 and 20:4n-6, were rather similar between diets and generations at approximately 12 and 0.5%, respectively. The n-3 fatty acids were markedly influenced by the diet. Linolenic acid (18:3n-3) was highest ($P < 0.05$) in the milk from the rats fed the vegetable oil diet compared with the other dietary groups, whereas the long-chain n-3 PUFA, 20:5n-3, 22:5n-3, and 22:6n-3, were found in greater concentrations in the marine oil-fed groups. The fish oil diet in the first generation resulted in higher ($P < 0.05$) levels of 20:5n-3 and 22:6n-3, and the seal oil diet resulted in the highest ($P < 0.05$) level of 22:5n-3. In the second generation, the levels of 20:5n-3 and 22:6n-3 in the milk from the seal and fish oil group were not different, but 22:5n-3 remained higher ($P < 0.05$) in the seal oil group.

The regiospecific distribution of fatty acids in the *sn*-2 position of milk TAG from the three dietary groups is shown in Table 3. Within each diet group, the TAG structures of the milk from the first and second generation were generally similar. Medium-chain fatty acids (MCFA) (8:0 and 10:0) and 18:1 were present in lower levels in the *sn*-2 position when

TABLE 2
Fatty Acid Composition of Milk Lipids in First and Second Generation[a]

	Seal oil diet		Fish oil diet		Vegetable oil diet	
Diet	First generation	Second generation	First generation	Second generation	First generation	Second generation
6:0	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.3 ± 0.2
8:0	5.8 ± 1.5^{a}	5.5 ± 1.5^{a}	6.5 ± 2.4^{a,b}	7.4 ± 2.3^{b}	6.6 ± 1.4^{a,b}	4.7 ± 2.0^{a}
10:0	9.7 ± 2.9	8.7 ± 1.9	8.6 ± 3.3	11.1 ± 3.0	9.8 ± 0.8	7.0 ± 2.7
12:0	4.8 ± 1.9^{b}	3.9 ± 1.1^{a}	3.2 ± 1.6^{a}	4.9 ± 1.2^{b}	4.3 ± 1.4^{a,b}	3.0 ± 1.0^{a}
14:0	5.2 ± 2.1^{b}	4.6 ± 1.3^{b}	3.5 ± 1.0^{a}	4.6 ± 0.8^{b}	3.2 ± 1.0^{a}	2.2 ± 0.7^{a}
16:0	14.9 ± 1.7	15.1 ± 2.3	14.7 ± 1.6	15.9 ± 1.5	14.7 ± 1.3	16.5 ± 1.8
16:1n-7	5.3 ± 1.7^{d}	6.5 ± 0.5^{e}	3.0 ± 0.5^{c}	3.2 ± 0.6^{c}	1.0 ± 0.6^{a}	1.8 ± 0.6^{b}
18:0	2.3 ± 0.5^{a}	2.5 ± 0.4^{a}	2.6 ± 0.4^{a,b}	2.6 ± 0.4^{a,b}	2.6 ± 0.2^{a,b}	3.0 ± 0.3^{b}
18:1n-7/9	25.6 ± 6.5^{a}	25.2 ± 2.4^{a}	28.5 ± 3.3^{a}	27.3 ± 3.6^{a}	34.9 ± 2.0^{b}	40.1 ± 3.9^{c}
18:2n-6	12.0 ± 1.6^{a,b}	12.3 ± 0.7^{a,b}	12.9 ± 1.6^{b}	10.9 ± 1.5^{a}	12.0 ± 1.1^{a,b}	12.1 ± 1.0^{a,b}
18:3n-3	2.1 ± 2.5^{b}	1.0 ± 0.1^{a}	0.7 ± 0.2^{a}	0.5 ± 0.2^{a}	8.3 ± 0.3^{d}	6.8 ± 0.9^{c}
20:1	2.6 ± 1.0^{c}	3.3 ± 0.5^{d}	1.3 ± 0.4^{b}	0.9 ± 0.2^{b}	0.2 ± 0.1^{a}	0.1 ± 0.1^{a}
20:4n-6	0.4 ± 0.2	0.5 ± 0.2	0.5 ± 0.2	0.4 ± 0.2	0.3 ± 0.1	0.5 ± 0.3
20:5n-3	3.4 ± 1.3^{b}	3.3 ± 0.6^{b}	5.2 ± 0.7^{c}	3.9 ± 0.7^{b}	0.7 ± 0.2^{a}	0.8 ± 0.4^{a}
22:5n-3	1.7 ± 0.6^{d}	2.1 ± 0.4^{e}	1.2 ± 0.4^{c}	0.8 ± 0.2^{b}	0.2 ± 0.2^{a}	0.2 ± 0.1^{a}
22:6n-3	2.9 ± 1.2^{b}	3.7 ± 0.7^{c}	5.9 ± 0.8^{d}	4.2 ± 0.7^{c}	0.2 ± 0.1^{a}	0.2 ± 0.2^{a}

[a]For each fatty acid: Values (mean) not bearing the same superscript letter are significantly different at $P < 0.05$. If no superscript appears, values are not different.; $n \geq 7$.

TABLE 3
Fatty Acid Composition of 2-Monoacylglycerols of Milk Lipids in First and Second Generation[a]

	Seal oil diet		Fish oil diet		Vegetable oil diet	
Diet	First generation	Second generation	First generation	Second generation	First generation	Second generation
6:0	0.3 ± .5	—	0.3 ± 0.3	—	0.3 ± 0.3	—
8:0	2.2 ± 2.7	2.2 ± 1.6	1.8 ± 1.5	2.6 ± 2.1	1.2 ± 1.0	1.8 ± 1.2
10:0	4.9 ± 3.9	6.5 ± 4.3	2.8 ± 2.1	8.5 ± 3.6	4.0 ± 2.5	5.9 ± 2.8
12:0	4.9 ± 3.2^{b}	5.4 ± 2.1^{b}	2.5 ± 1.7^{a}	5.9 ± 1.3^{b}	4.1 ± 1.7^{a,b}	4.0 ± 1.4^{a,b}
14:0	9.0 ± 3.2^{b}	9.4 ± 2.3^{b}	5.6 ± 1.7^{a}	8.1 ± 1.2^{b}	5.3 ± 1.7^{a}	4.0 ± 1.0^{a}
16:0	33.3 ± 6.7	33.5 ± 4.7	31.7 ± 5.5	33.5 ± 4.5	32.0 ± 4.4	36.5 ± 4.9
16:1n-7	9.9 ± 1.3^{d}	10.8 ± 1.5^{e}	5.9 ± 0.7^{c}	5.6 ± 0.7^{c}	2.0 ± 0.7^{a}	3.3 ± 0.9^{b}
18:0	0.8 ± 0.2^{a,b}	0.5 ± 0.3^{a}	1.4 ± 0.6^{a,b}	0.6 ± 0.3^{a}	1.3 ± 0.4^{a,b}	1.6 ± 1.4^{b}
18:1n-7/9	12.9 ± 2.8^{a}	12.1 ± 1.8^{a}	19.9 ± 3.2^{b}	14.5 ± 2.5^{a}	23.2 ± 3.5^{c}	20.7 ± 2.1^{b,c}
18:2n-6	11.0 ± 1.8^{a}	10.7 ±1.4^{a}	13.7 ± 1.4^{b}	10.4 ± 1.4^{a}	13.0 ± 1.3^{b}	11.1 ± 1.2^{a}
18:3n-3	1.4 ± 0.5^{a}	1.1 ± 0.3^{a}	1.3 ± 0.6^{a}	0.8 ± 0.2^{a}	9.8 ± 1.2^{c}	7.8 ± 1.2^{b}
20:1	0.6 ± 0.7^{b}	0.3 ± 0.3^{a,b}	0.5 ± 0.6^{b}	0.0 ± 0.0^{a}	0.2 ± 0.2^{a,b}	0.0 ± 0.0^{a}
20:4n-6	0.4 ± 0.2^{a,b}	0.3 ± 0.1^{a}	0.5 ± 0.2^{a,b}	0.4 ± 0.1^{a,b}	0.4 ± 0.3^{a,b}	0.8 ± 0.5^{b}
20:5n-3	1.8 ± 1.0^{c}	1.2 ± 0.4^{b,c}	2.8 ± 1.2^{d}	1.7 ± 0.3^{c}	0.9 ± 0.6^{a,b}	0.3 ± 0.1^{a}
22:5n-3	2.0 ± 0.9^{d}	1.5 ± 0.5^{c}	1.2 ± 0.4^{b,c}	0.9 ± 0.1^{a,b}	0.8 ± 0.4^{a,b}	0.4 ± 0.2^{a}
22:6n-3	3.1 ± 0.9^{b}	3.0 ± 0.6^{b}	5.5 ± 1.4^{c}	5.1 ± 0.5^{c}	0.6 ± 0.3^{a}	0.4 ± 0.3^{a}

[a]For each fatty acid: Values (mean) not bearing the same superscript letter are significantly different at $P < 0.05$. If no superscript appears, values are not different; n ≥ 7, mole% calculated from GLC analysis of methyl esters. See Table 1 for abbreviations

compared with the total level in milk TAG (Table 2), whereas 14:0, 16:0, and 16:1 were present at higher levels in the *sn*-2 position. When present, 20:1 was mostly located in the *sn*-1/*sn*-3 positions. The contents of 18:2n-6 and 20:4n-6 were similar in the total TAG and in *sn*-2 position, and only small differences between diets and generations were found; 20:5n-3 was enriched ($P < 0.05$) in the milk from the marine groups, but it was mostly located in the *sn*-1/*sn*-3 positions, as seen from the low contents in the *sn*-2 position. On the other hand, 22:6n-3 appeared to be enriched in the *sn*-2 position in the second generation of the fish oil group, and in the seal oil group, 22:5n-3 was significantly enriched ($P < 0.05$) in the *sn*-1/*sn*-3 positions in the second generation.

DISCUSSION

Maternal milk TAG supply the infant with MCFA (13) for readily absorbable energy and n-6 as well as n-3 PUFA (14), which are important for membrane function and neural development. Furthermore, milk possesses a particular TAG structure with 16:0 mostly located in the *sn*-2 position (15). The fatty acids in milk have three different origins: the diet, adipose stores, and *de novo* synthesis in the mammary glands (16). In healthy women eating a Western diet, *de novo* synthesis accounts for 10–12% of the total fat in milk, and the diet for approximately 30% (16).

In this experiment we examined the effect of maternal dietary fatty acid profile and TAG structure on the fatty acid profile and the milk fat TAG structure in a two-generation experiment with rats. The diets were rich in n-3 PUFA, either from fish oil, from seal oil, or from vegetable oil, to elucidate the effect of dietary pre-formed 22:6n-3 vs. 18:3n-3 and the effect of dietary TAG structure. The two-generation model was used to study (i) if an adaptation to an intake of a high level of 18:3n-3 was followed by increased formation of 22:6n-3 and (ii) if a possible effect of TAG structure of the marine fat was increased or decreased in the second generation.

In rat milk we found up to 12% of long-chain n-3 PUFA following intake of marine fats, which is considerably higher than reported for chow-fed rats at about 1.2% (17) or human milk at about 3% (18). The milk from the rats fed the vegetable oil diet contained 7–8% 18:3n-3 and only a small amount (approximately 1%) of long-chain n-3 PUFA, indicating that although the desaturation and elongation of 18:3n-3 to 22:6n-3 in the liver are very active in the rat (19) this did not result in much higher levels of these n-3 PUFA in milk. Furthermore, the contents of 20:5n-3 and 22:6n-3 in the milk of the vegetable oil-fed rats did not increase from the first to the second generation as it did in the heart (4). Therefore, in order to obtain the higher levels of 22:6n-3 in the milk, this fatty acid needs to be included in the maternal diet.

The incorporation of n-3 PUFA into milk lipids (Table 2) showed some significant differences in content, but not positional distribution (Table 3), between the two marine oil groups in the first and second generation. A direct comparison between each of the n-3 PUFA was not possible, since there were differences between the two diets. However, the total n-3 PUFAs in the diets was more similar (fish oil 20.6%; seal oil 19.1%). The results clearly showed significantly higher incorporation ($P < 0.05$) of the n-3 PUFA into milk of fish oil-fed rats compared to seal oil-fed rats in the first generation (12.3 vs. 8.0%). This difference could not have occurred during absorption because the differences in TAG structure were previously shown not to affect the absorption of these two oils (20). A more probable explanation of these results may be at the level of chylomicron metabolism, since we have shown that the chylomicrons are cleared at different rates for these marine oils (7). In the second generation, there

were no significant differences in the n-3 PUFA between these two dietary marine groups (8.9 vs. 9.1%), which may suggest an adaptation. Certainly, there are strong trends to maintain a rather uniform fatty acid profile in milk fatty acid composition.

Furthermore, it is evident from these results that the positional distribution of fatty acids on dietary marine oil TAG was not preserved (Table 3) during milk synthesis as it is during fat absorption. The tailoring of TAG compared to the dietary TAG indicates that milk TAG are thus mostly synthesized *de novo* by the phosphatidic acid pathway, and the distribution of fatty acids in the milk TAG molecule, therefore, reflects the proportion of substrate fatty acids and specificity of the transacylases, and not the dietary TAG structure. In agreement with this, milk from seals eating large amounts of fish with 22:6n-3 in the *sn*-2 position of the TAG does not contain 22:6n-3 in the *sn*-2 position of the TAG (21,22).

The levels of total MCFA (6:0 to 14:0) in the milk ranged from 17 to 28% despite their absence in all the diets. Continued exposure to these diets into the second generation resulted in an increase in MCFA in the fish oil group and a decrease in the vegetable oil group. Previous studies of milk from rats fed chow diet (17) showed that MCFA (6:0 to 14:0) comprised approximately 20% in agreement with our vegetable oil group. The level of MCFA in human milk is between 5 and 10% (13,16,23). The majority of these are synthesized in the mammary gland, the synthesis being depressed by a fat-rich diet and increased by a carbohydrate-rich diet (24).

The contents of 20:4n-6 in the milk from the three groups were all similar and low (0.3–0.5%). Except for seal oil, which contained only 0.1%, none of the other diets contained 20:4n-6. The contents of 18:2n-6 (11–12%) indicated the availability of the precursor, but the higher level of 18:2n-6 in the vegetable oil did not result in elevated levels in the milk. This may suggest a regulation of the amount of long-chain PUFA, such as 20:4n-6 or 22:6n-3 in the milk, when these fatty acids are not directly available from the diet as also pointed out for human milk (14).

Palmitic acid is dominant in the *sn*-2 position and 18:1 in the *sn*-1/*sn*-3 position, and only 18:1 was affected by diet. This indicates that the TAG structure is strongly conserved throughout the two generations irrespective of diet. Milk TAG from several species (25), including humans (26), consistently show a similar pattern of 16:0 and 18:1 distribution. Palmitic acid in the *sn*-2 position was shown to be better absorbed than when present in the *sn*-1/*sn*-3 positions (15) which assures a more efficient transfer of 16:0 to the neonate.

Numerous studies have shown that the amount of n-3 fatty acids in the postnatal diet is very important for normal development of the central nervous system (1,27,28). The presence of 22:6n-3 seems especially vital, since the absence of this particular fatty acid in brain and retina has been correlated to reduced learning ability (29) and decreased visual acuity (30). Recent reports have questioned the necessity for preformed 22:6n-3 in the infant diet, indicating that term infants can convert 18:3n-3 into 22:6n-3 at a sufficient rate to support visual function (31). Others (32–34) found that 20:5n-3 and 22:6n-3 at 0.3 and 0.2% of dietary fatty acids in newborn infants secured an amount of long-chain n-3 fatty acids in the red blood cell phospholipids similar to what is found in human milk-fed infants. Infants given a supplement of n-3 fatty acids showed higher levels of 20:5n-3 and 22:6n-3 in the red blood cells at the expense of 20:4n-6 and accompanying growth retardation (32).

Our study has demonstrated that even under considerable manipulation of the maternal diet for two generations the structure of the milk TAG is highly conserved, and that the milk secretory system preserves the overall fatty acid profile and includes easily digested MCFA into the maternal milk. However, if a high level of long-chain n-3 fatty acids in the milk is desired, these fatty acids must be present in the maternal diet, since no adaptation to 18:3n-3 as a dietary source of long chain n-3 fatty acids was observed in this two-generation experiment.

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New Unsaturated Long-Chain Fatty Acids in the Phospholipids from the Axinellida Sponges *Trikentrion loeve* and *Pseudaxinella* cf. *lunaecharta*

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ABSTRACT: In order to identify new structures and especially those involved as biosynthetic intermediates, the fatty acid composition of whole phospholipids from two Senegalese marine sponges from the order Axinellida, *Trikentrion loeve* and *Pseudaxinella* cf. *lunaecharta*, has been investigated by analytical gas chromatography and gas chromatography/mass spectrometry. Several new fatty acids were identified as methyl esters and *N*-acyl pyrrolidides, namely 16-eicosenoic, 11-tetracosenoic, 5-pentacosenoic, 11-hexacosenoic, 11-octacosenoic, 23-triacontenoic, 17,21-hexacosadienoic, 19,23-octacosadienoic, 9,23-triacontadienoic, 5,9,21-hexacosatrienoic, and 5,9,25-triacontatrienoic. *Trikentrion loeve* and *P.* cf. *lunaecharta* contain fifteen (25.7% of the total acid mixture) and thirteen (30.4%) Δ5,9 fatty acids, respectively. Based on gas chromatography/Fourier transform infrared experiments, the double bonds were assigned the (*Z*) configuration. Biosynthesis of dienoic and trienoic demospongic acids possessing an n-5 or an n-7 terminal double bond is discussed.
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As part of our continuing investigation of phospholipids from marine sponges of the Senegalese coast (1–5), we recently reported the occurrence of three new C_{30} unsaturated fatty acids in the sponge *Trikentrion loeve* Carter (3). The novel fatty acids 23-triacontenoic [**3**] and 9,23-triacontadienoic [**6**] are biosynthetic intermediates of the known 5,9,23-triacontatrienoic acid, whereas the novel isomeric acid 5,9,25-triacontatrienoic [**8**] involves an unusual pathway. Consequently, we undertook a more extensive study of this phospholipid fraction, together with an additional phospholipid fraction from the Senegalese sponge *Pseudaxinella* cf. *lunaecharta*, also belonging to the order Axinellida.

Since the findings of Litchfield and co-workers (6,7), sponge phospholipids have been known to contain high levels of very long-chain fatty acids (23–34 carbon atoms), including specific acids possessing unique 5(*Z*), 9(*Z*)-diunsaturation and sometimes an additional double bond near the chain terminus. The biosynthesis of these sponge fatty acids has been investigated, but a number of postulated intermediates have not yet been identified as natural products (8–10). The Senegalese sponge *Higginsia tethyoides* (order Axinellida, family Axinellidae) contains an unusually large amount (34.2%) of 5,9,23-triacontatrienoic acid and a new class of 2-methoxy fatty acids (11). In our search for Δ5,9 demospongic acids and their metabolic intermediates, we recently investigated the New Caledonian sponge *Cinachyrella* aff *schulzei*, which contained the largest number of Δ5,9 demospongic acids (15 acids) encountered to date in a marine sponge (4). In the latter sponge, we observed for the first time the occurrence of 27-methyl-5,9-octacosadienoic acid, which was also recently identified in two Caribbean sponges (12).

This report details the fatty acid composition of whole phospholipids from *T. loeve* and *P.* cf. *lunaecharta*, and describes the characterization of several new unsaturated demospongic acids presented in Figure 1.

EXPERIMENTAL PROCEDURES

Sponge material, extraction, and isolation of phospholipids. Specimens of *T. loeve* Carter and *P.* cf. *lunaecharta* were respectively collected by hand (scuba-diving) at Thiouri-Ba (40–45 m depth, off Dakar) and at Pointe Senti (100 km south of Dakar, ~1 m depth) in February of 1990. The sponges were washed in sea water, carefully cleaned, ground in a Waring blender with chloroform/methanol (1:1, vol/vol) and steeped twice in this solvent for 24 h at room temperature. The combined extracts yielded the crude total lipids. Phospholipids were separated from other lipids by column chromatography on silica gel (70–230 mesh) with hexane, chloroform, acetone, and methanol (phospholipid fraction) as successive eluents. The phospholipid mixture was analyzed by thin-layer chromatography (TLC) on silica gel (chloroform/methanol/water, 65:25:4, by vol). Standard phospholipid samples and normal, odd, and even (from C_{20} to C_{31}) fatty acids methyl esters (FAME) were purchased from the Sigma Chemical Co. (St. Louis, MO). The phospholipid mixture from *T.*

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Abbreviations: ECL, equivalent chain length; FAME, fatty acid methyl esters; GC, gas–liquid chromatography; GC/FTIR, GC/Fourier transform infrared; GC/MS, GC/mass spectrometry; RT, retention time; TLC, thin-layer chromatography; 3,7,11,15-TMHD, 3,7,11,15-tetramethylhexadecanoic; 4,8,12-TMTD, 4,8,12-trimethyltridecanoic.

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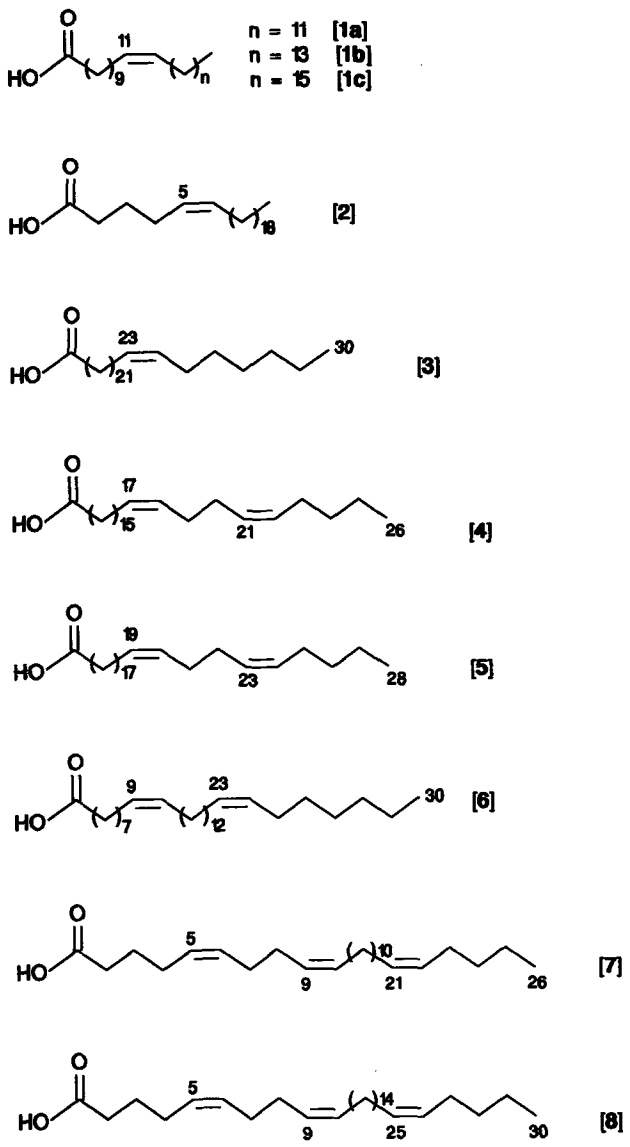


FIG. 1. New demospongic fatty acids from *Trikentrion loeve* and *Pseudaxinella* cf. *lunaecharta*.

loeve also was studied by ^{31}P nuclear magnetic resonance (NMR) performed on a AC-400 Bruker NMR spectrometer (Karlsruhe, Germany) at 161.977 MHz. The phospholipid sample was dissolved in deuterated chloroform/methanol (2:1, vol/vol), and 85% phosphoric acid was used as external reference.

Gas–liquid chromatography (GC) and GC/mass spectrometry (GC/MS) analyses. The phospholipid fatty acids were converted to methyl esters by reaction (30 min under reflux) with methanolic hydrogen chloride, dissolved in hexane, and purified by column chromatography (silica gel, hexane/diethyl ether, 10:1, vol/vol). The resulting methyl esters were analyzed by GC. *N*-acyl pyrrolidide derivatives were prepared by direct treatment of methyl esters with pyrrolidine/acetic acid (10:1, vol/vol) under reflux (2 h) and purified by TLC (silica gel, hexane/diethyl ether, 1:2, vol/vol). FAME were hydrogenated by stirring (4 h) at room pressure and temperature in methanol with catalytic amounts of platinum (IV) oxide (Adam's catalyst; Sigma-Aldrich Chimie, Saint-Quentin-Fallavier, France). Methyl esters were analyzed by GC using a Carlo-Erba 4130 chromatograph (Milano, Italy) and a nonpolar OV-1 silica capillary column (25 m × 0.32 mm i.d., 0.40 μm phase thickness); hydrogen was used as carrier gas (50 kPa, split 5/100). The temperature was programmed at 3°C/min, from 180 to 300°C. Combined GC/MS was performed on a Hewlett-Packard HP-5890 chromatograph linked to an HP 9000/345 integrator (Palo Alto, CA). The GC column was a 30 m × 0.32 mm i.d. fused silica capillary column coated with DB-1 (0.25 μm phase thickness). The carrier gas was helium (0.5 bar, split 1/30). Column temperature was programmed from 180 to 310°C, at 3°C/min, for both derivatives (methyl esters and pyrrolidides).

GC/Fourier transform infrared (FTIR). GC/FTIR spectra were recorded with a 20SxB Nicolet spectrometer (Madison, WI), interfaced with a 5300 Mega Carlo Erba FID chromatograph equipped with a fused-silica capillary column (DB-1; 60 m, 0.32 mm i.d., 0.25 μm film thickness; J&W Scientific, Folson, CA). Oven temperature was programmed from 70 to 130°C (15°C/min) and from 130 to 290°C (4°C/min); isotherm steps of 2 min at 70°C and 40 min at 290°C were used. The carrier gas was helium (140 kPa). Pressure of hydrogen and air was 50 and 100 kPa, respectively. The light-pipe (infrared gas cell) was gold-coated, with dimensions of 160 × 1.2 mm. Temperature was maintained at 293°C for the experiments. Vapor phase infrared spectra were performed with 8 cm^{-1} resolution, and 16 data scans were collected and co-added per data file (2 s for each spectrum). Chromatogram reconstruction was performed with the Gram-Schmidt algorithm.

RESULTS

As determined by the chloroform/methanol extraction procedure, the sponge *P.* cf. *lunaecharta* (dry weight) yielded 2.3% of total lipids, and the sponge *T. loeve* yielded an unusual value of 8.0%. Phospholipids (215 mg) were recovered from 241 g (dry weight) of *P.* cf. *lunaecharta* (3.8% of total lipids), while 94 g of *T. loeve* (dry weight) yielded 192 mg (2.5%). As shown by TLC and ^{31}P NMR spectroscopy, the main phospholipid classes from *T. loeve* were phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylglycerol. As shown by TLC, the main phospholipid classes from *P.* cf. *lunaecharta* were phosphatidylethanolamine, phosphatidylserine, and phosphatidylglycerol. The only whole phospholipid fractions isolated by column chromatography (methanol-eluted fractions) were studied. The phospholipid fatty acid composition of both sponges is shown in Table 1, and the new demospongic acids are represented in Figure 1.

More than sixty fatty acids were identified in *T. loeve* or *P.* cf. *lunaecharta* by capillary GC of their methyl esters and GC/MS of their methyl esters and *N*-acyl pyrrolidides. Very

TABLE 1
Phospholipid Fatty Acids from Two Senegalese Marine Sponges of Order Axinellida[a,b]

Fatty acids	ECL	Abundance in weight (%) *Trikentrion loeve*	*Pseuchaxinella* cf. *lunaecharta*
Dodecanoic (12:0)	12.00	0.8	0.8
Tetradecanoic (14:0)	14.00	3.7	2.1
4,8,12-Trimethyltridecanoic (*br*-16:0)	14.52	12.4	3.1
13-Methyltetradecanoic (*i*-15:0)	14.65	0.4	0.7
12-Methyltetradecanoic (*ai*-15:0)	14.72	0.4	0.6
Pentadecanoic (15:0)	15.00	0.8	0.9
14-Methylpentadecanoic (*i*-16:0)	15.66	0.7	3.5
8-Hexadecenoic (16:1)	15.73	4.2	—
9-Hexadecenoic (16:1)	15.77	—	7.6
Hexadecanoic (16:0)	16.00	11.2	6.8
7-Methyl-6-hexadecenoic (*br*-17:1)	16.36	2.7	0.6
15-Methylhexadecanoic (*i*-17:0)	16.66	1.2	1.2
14-Methylhexadecanoic (*ai*-17:0)	16.73	1.0	1.1
9-Heptadecenoic (17:1)	16.77	0.5	1.0
Heptadecanoic (17:0)	17.00	0.8	0.6
9,12-Octadecadienoic (18:2)	17.58	1.2	0.3
16-Methylheptadecanoic (*i*-18:0)	17.66	4.3	1.1
3,7,11,15-Tetramethylhexadecanoic (*br*-20:0)	17.72	2.2	0.7
9-Octadecenoic (18:1)	17.77	4.4	1.1
11-Octadecenoic (18:1)	17.80	1.3	0.9
Octadecanoic (18:0)	18.00	5.2	1.8
12-Methyloctadecanoic (*br*-19:0)	18.40	—	0.8
15-Methyloctadecanoic (*br*-19:0)[c]	18.42	0.6	—
6-Nonadecenoic (19:1)	18.72	0.3	1.2
12-Nonadecenoic (19:1)	18.80	0.3	1.2
Nonadecanoic (19:0)	19.00	0.5	trace
11-Eicosenoic (20:1)	19.70	0.9	0.7
13-Eicosenoic (20:1)	19.78	0.7	0.6
14-Eicosenoic (20:1)[d]	19.80	—	0.5
16-Eicosenoic (20:1)[d]	19.90	0.2	—
Eicosanoic (20:0)	20.00	0.5	1.8
Heneicosanoic (21:0)	21.00	0.3	—
11-Docosenoic (22:1)	21.68	0.3	0.6
15-Docosenoic (22:1)	21.74	0.8	0.9
Docosanoic (22:0)	22.00	0.5	1.4
Tricosanoic (23:0)	23.00	trace	0.8
11-Tetracosenoic (24:1)[c] **[1a]**	23.66	—	0.6
15-Tetracosenoic (24:1)	23.75	—	4.3
17-Tetracosenoic (24:1)	23.80	1.5	3.7
Tetracosanoic (24:0)	24.00	0.6	3.2
5-Pentacosenoic (25:1)[c] **[2]**	24.53	—	0.7
16-Pentacosenoic (25:1)	24.88	0.2	0.9
Pentacosanoic (25:0)	25.00	1.1	trace
11-Hexacosenoic (26:1)[c] **[1b]**	25.64	—	1.9
17,21-Hexacosadienoic (26:2)[d] **[4]**	25.70	—	1.5
17-Hexacosenoic (26:1)	25.78	1.2	1.3
19-Hexacosenoic (26:1)	25.81	1.8	0.6
11-Octacosenoic (28:1)[c] **[1c]**	27.62	—	0.8
19,23-Octacosadienoic (28:2)[d] **[5]**	27.66	—	1.3
21-Octacosenoic (28:1)	27.80	1.1	—
9,23-Triacontadienoic (30:2)[e] **[6]**	29.58	0.2	—
23-Triacontenoic (30:1)[e] **[3]**	29.69	0.5	—
Δ5,9-Demospongic acids[f]			
5,9-Tetracosadienoic (24:2)	23.56	—	0.8
23-Methyl-5,9-tetracosadienoic (*i*-25:2)	24.12	—	1.8
5,9,19-Hexacosatrienoic (26:3)	25.12	—	0.2
5,9,21-Hexacosatrienoic (26:3)[d] **[7]**	25.24	—	2.3
5,9-Hexacosadienoic (26:2)	25.54	1.4	20.3
25-Methyl-5,9-hexacosadienoic (*i*-27:2)	26.18	0.4	1.1
24-Methyl-5,9-hexacosadienoic (*ai*-27:2)	26.28	0.7	0.6

Continued on next page

TABLE 1 *(continued)*

Fatty acids	ECL	Abundance in weight (%) *Trikentrion loeve*	*Pseuchaxinella* cf. *lunaecharta*
5,9-Heptacosadienoic (27:2)	26.52	0.4	1.1
26-Methyl-5,9-heptacosadienoic (*i*-28:2)	27.14	0.2	—
5,9,19-Octacosatrienoic (28:3)	27.25	0.4	—
5,9,21-Octacosatrienoic (28:3)	27.33	0.7	0.6
5,9,23-Octacosatrienoic (28:3)	27.39	—	0.5
5,9-Octacosadienoic (28:2)	27.54	2.3	0.2
27-Methyl-5,9-octacosadienoic (*i*-29:2)	28.12	0.2	—
26-Methyl-5,9-octacosadienoic (*ai*-29:2)	28.22	0.2	—
5,9-Nonacosadienoic (29:2)	28.49	0.9	—
5,9,23-Triacontatrienoic (30:3)	29.38	14.8	0.9
5,9,25-Triacontatrienoic (30:3)[e] **[8]**	29.46	2.1	—

[a]ECL, equivalent chain length (DB1); *i*, *iso*; *ai*, anteiso; *br*, branched; trace, <0.1%.
[b]Identified minor fatty acids (<0.2%): 10:0, *i*-19:0 (18.66), *ai*-19:0 (18.73), 20:4n-6 (19.20), *i*-20:0 (19.65), *ai*-20:0 (19.72), *ai*-21:0 (21.73), 23:0, *i*-27:0 (26.64), 27:0, 28:0, 30:0.
[c]First reported in phospholipids.
[d]Not previously found in nature.
[e]First reported in *T. loeve* (see Ref. 3).
[f]Detected Δ5,9 demospongic acids for which the location of the terminal double bond was not determined: Δ5,9,x-27:3 (26.32, trace) in *P.* cf. *lunaecharta*; Δ5,9,y-29:3 (28.27, 0.5%) and Δ5,9,z-29:3 (28.30, 0.5%) in *T. loeve*.

long-chain acids (>24 carbon atoms) accounted for about 34% in *T. loeve* and 52% in *P.* cf. *lunaecharta*. Most of the fatty acids compiled in Table 1 were identified as methyl esters by comparing their equivalent chain length (ECL) values with those of commercial standards and other known compounds. Capillary GC analysis of a hydrogenated aliquot also provided useful information for detection and identification of fatty acids. For many components, *N*-acyl pyrrolidide GC/MS data allowed us to confirm the structures and to determine the double bond and branching positions. GC retention times and mass spectral data of selected fatty acid pyrrolidides are as follows.

12-Methyloctadecanoic acid pyrrolidide [retention time (RT): 22.44 min]. Mass spectrum (MS) *m/z* (relative intensity): 351 (M^+, 1.2), 336 (0.7), 322 (0.3), 308 (0.8), 294 (0.3), 280 (1.2), 266 (1.2), 238 (1.6), 224 (0.8), 210 (0.8), 196 (0.9), 182 (1.6), 168 (2.3), 154 (1.3), 140 (2.5), 126 (14.8), 113 (100), 98 (9.4), 84 (4.7), 71 (12.5), 70 (12.1), 55 (17.2), 43 (23.4), 41 (11.0).

15-Methyloctadecanoic acid pyrrolidide (RT: 22.98 min). MS *m/z* (relative intensity): 351 (M^+, 0.6), 336 (0.2), 322 (0.4), 308 (0.7), 294 (<0.1), 280 (0.5), 266 (0.1), 252 (0.2), 238 (0.3), 224 (0.3), 210 (0.2), 196 (0.4), 182 (0.8), 168 (1.2), 154 (0.7), 140 (1.6), 126 (13.4), 113 (100), 98 (7.5), 84 (6.0), 71 (16.0), 70 (15.9), 55 (28.1), 43 (47.6).

14-Eicosenoic acid pyrrolidide (RT: 25.91 min). MS *m/z* (relative intensity): 363 (M^+, 9.9), 348 (1.1), 334 (3.4), 320 (3.3), 306 (4.8), 292 (4.6), 278 (3.5), 266 (6.3), 253 (4.3), 238 (2.4), 224 (2.8), 210 (2.1), 196 (4.3), 195 (5.3), 182 (4.2), 168 (4.5), 154 (4.3), 140 (6.0), 126 (33.9), 113 (100), 98 (43.5), 81 (95.0), 75 (95.0), 71 (55.0), 70 (55.0), 55 (100), 43 (96.0), 41 (100).

16-Eicosenoic acid pyrrolidide (RT: 26.01 min). MS *m/z* (relative intensity): 363 (M^+, 5.7), 348 (0.2), 334 (2.7), 320 (2.7), 306 (2.2), 295 (2.2), 294 (0.8), 281 (4.6), 280 (0.8), 266 (4.1), 253 (2.1), 252 (2.1), 239 (1.3), 238 (1.4), 224 (2.7), 210 (2.3), 196 (3.1), 195 (2.9), 182 (2.9), 168 (4.0), 154 (1.8), 140 (5.3), 126 (38.4), 113 (100), 98 (19.2), 84 (21.3), 75 (85.0), 72 (38.6), 70 (40.3), 55 (100), 43 (84.9).

*5-Pentacosenoic acid pyrrolidide [**2**] (RT: 36.07 min).* MS *m/z* (relative intensity): 433 (M^+, 2.7), 418 (2.0), 404 (0.1), 390 (0.7), 376 (0.6), 362 (1.5), 348 (0.9), 336 (0.7), 334 (0.9), 320 (1.4), 306 (1.2), 292 (1.4), 278 (1.6), 264 (1.9), 250 (3.6), 236 (3.9), 222 (3.2), 208 (3.2), 196 (2.3), 194 (2.6), 182 (4.8), 180 (5.1), 166 (3.6), 164 (3.0), 154 (1.8), 152 (7.8), 140 (4.7), 126 (35.9), 113 (100), 98 (20.3), 85 (9.4), 83 (9.2), 72 (29.7), 70 (28.6), 69 (25.0), 55 (42.2), 43 (31.2), 41 (21.9).

*11-Hexacosenoic acid pyrrolidide [**1b**] (RT: 38.36 min).* MS *m/z* (relative intensity): 447 (M^+, 6.8), 432 (0.3), 418 (0.5), 404 (1.0), 390 (0.3), 376 (0.3), 362 (0.4), 348 (0.5), 346 (0.4), 334 (0.5), 320 (0.7), 306 (0.9), 292 (1.2), 278 (1.7), 264 (2.4), 250 (4.1), 236 (4.5), 224 (2.4), 210 (2.1), 196 (2.0), 194 (1.6), 182 (6.2), 180 (5.3), 168 (4.3), 154 (2.0), 140 (4.7), 126 (39.1), 113 (100), 98 (17.2), 85 (7.8), 72 (28.1), 70 (17.1), 55 (31.3), 43 (29.7), 41 (15.6).

*11-Octacosenoic acid pyrrolidide [**1c**] (RT: 41.92 min).* MS *m/z* (relative intensity): 475 (M^+, 3.0), 460 (0.2), 446 (0.2), 443 (0.1), 432 (0.1), 418 (0.2), 404 (0.3), 390 (0.2), 376 (0.5), 362 (0.3), 348 (0.8), 334 (0.7), 320 (1.2), 306 (1.5), 292 (1.3), 278 (2.8), 265 (1.6), 264 (2.7), 250 (1.9), 238 (1.4), 236 (1.6), 224 (1.6), 222 (1.4), 210 (3.0), 209 (2.2), 196 (2.5), 182 (3.6), 168 (3.8), 155 (2.2), 154 (2.5), 140 (3.1), 126 (28.1), 113 (100), 98 (17.2), 85 (7.8), 81 (7.2), 72 (25.8), 70 (25.2), 55 (31.2), 43 (31.2).

17,21-Hexacosadienoic acid pyrrolidide [4]. (RT: 38.60 min). MS *m/z* (relative intensity): 445 (M^+, 12.0), 430 (0.2), 416 (0.8), 402 (1.4), 388 (1.6), 376 (0.7), 374 (0.9), 362 (1.6), 360 (0.8), 348 (4.7), 334 (4.4), 322 (3.0), 320 (6.5), 308 (4.5), 306 (4.3), 294 (2.5), 280 (2.0), 266 (2.1), 252 (2.2), 238 (2.3), 224 (2.2), 210 (2.1), 196 (2.2), 182 (3.5), 180 (3.4), 168 (3.6), 155 (1.7), 154 (1.9), 140 (4.0), 127 (8.0), 126 (35.9), 113 (100), 98 (18.0), 84 (10.9), 81 (7.8), 72 (43.7), 70 (57.8), 55 (46.1), 43 (31.2), 41 (23.4).

19,23-Octacosadienoic acid pyrrolidide [5]. (RT: 42.21 min). MS *m/z* (relative intensity): 473 (M^+, 6.4), 459 (<0.1), 444 (0.5), 430 (0.8), 416 (0.9), 402 (0.7), 390 (0.9), 388 (0.8), 376 (3.9), 362 (2.7), 348 (1.4), 336 (1.2), 322 (2.1), 308 (1.6), 294 (1.8), 280 (2.2), 266 (2.2), 252 (2.0), 238 (2.2), 224 (2.0), 210 (2.2), 196 (2.7), 182 (3.3), 180 (3.9), 168 (3.7), 155 (1.8), 154 (2.3), 140 (4.3), 126 (32.8), 113 (100), 98 (20.3), 84 (7.8), 81 (12.5), 72 (43.7), 70 (57.8), 55 (42.2), 43 (28.1), 41 (18.7).

26-Methyl-5,9-octacosadienoic acid pyrrolidide (RT: 42.87 min). MS *m/z* (relative intensity): 487 (M+, 0.9), 473 (0.4), 458 (0.3), 452 (0.4), 448 (0.8), 430 (0.7), 417 (0.3), 360 (0.6), 350 (0.3), 276 (1.6), 266 (0.6), 250 (1.7), 234 (1.3), 226 (0.8), 207 (2.0), 196 (0.3), 194 (0.3), 192 (0.9), 180 (24.5), 166 (4.1), 152 (1.8), 140 (3.6), 126 (19.9), 113 (100), 98 (27.0), 85 (11.2), 71 (13.2), 55 (27.2), 43 (26.8).

5,9,21-Hexacosatrienoic acid pyrrolidide [7] (RT: 37.15 min). MS *m/z* (relative intensity): 443 (M^+, 3.1), 434 (0.2), 428 (0.1), 414 (0.2), 400 (0.2), 386 (0.3), 372 (0.5), 360 (0.3), 346 (0.2), 332 (0.2), 318 (0.6), 304 (0.2), 290 (0.4), 276 (0.3), 262 (0.4), 260 (0.7), 248 (0.7), 247 (0.5), 234 (1.1), 220 (0.9), 206 (0.5), 194 (0.5), 180 (18.0), 166 (0.8), 152 (1.0), 140 (1.6), 126 (14.8), 113 (100), 98 (14.1), 85 (7.0), 72 (16.4), 71 (16.1), 70 (8.2), 55 (25.0), 43 (18.8), 41 (12.5).

Saturated fatty acids. In *P.* cf. *lunaecharta*, a relatively short-chain acid was readily identified as its methyl ester showed an ECL value of 18.42 and a molecular ion (GC/MS) at *m/z* 312 accompanied by major common fragmentation ions at *m/z* 74 (base peak) and *m/z* 87, indicating a branched nonadecanoic structure. This compound remained unchanged upon catalytic hydrogenation. The MS of the corresponding pyrrolidide confirmed a nonadecanoic structure (19:0), showing a molecular ion peak at *m/z* 351. Enhanced peaks at *m/z* 280 (C_{14} fragment) and *m/z* 308 (C_{16}), and a very low fragmentation ion at *m/z* 294 (C_{15}) indicated a methyl branch at C-15. Thus, we characterized the 15-methyloctadecanoic acid previously found only in total lipids from the sponge *Dysidea fragilis*, but not described in detail (13). In the same manner, the unusual 12-methyloctadecanoic acid known since 1975 and recently described from *Agelas conifera* (14) was identified. Both MS of the pyrrolidide derivatives are given here. Two isoprenoid fatty acids were readily identified as methyl esters from GC and GC/MS data, namely 4,8,12-trimethyltridecanoic (4,8,12-TMTD) and 3,7,11,15-tetramethylhexadecanoic (3,7,11,15-TMHD, phytanic acid) (15,16).

Monoenoic fatty acids. Several new phospholipid long-chain monoenoic acids were identified in these sponges. 23-Triacontenoic acid **[3]**, characterized in our previous study (3) and found in *T. loeve*, is the longest monoenoic acid yet isolated from a sponge. Another unusual acid detected in *P.* cf. *lunaecharta* was readily identified as its methyl ester and had an ECL value of 24.53 and a molecular ion peak at *m/z* 394, indicating a 25:1 acid structure. The pyrrolidide derivative gave a molecular ion peak at *m/z* 433, indicating a 25:1 structure, and this peak disappeared upon catalytic hydrogenation. Key fragmentation ions at *m/z* 140 (C_4 fragment) and *m/z* 152 (C_5) indicated Δ5 unsaturation. Thus, we identified 5-pentacosenoic acid **[2]**, to our knowledge not previously described in any phospholipid fraction. Both new monoenoic short-chain acids, 16-eicosenoic in *T. loeve* and 14-eicosenoic in *P.* cf. *lunaecharta*, were also identified. As their methyl esters, these acids had ECL values of 19.90 and 19.80, respectively, and their MS displayed a molecular ion peak at *m/z* 324, suggesting a 20:1 acid structure. Both GC peaks disappeared upon catalytic hydrogenation and both MS of their pyrrolidides exhibited a molecular ion peak at *m/z* 363 (20:1 structure). Key fragments at *m/z* 294 (C_{15}) and *m/z* 306 (C_{16}) indicated Δ16 unsaturation for the first acid, whereas those at *m/z* 266 (C_{13}) and *m/z* 278 (C_{14}) indicated Δ14 unsaturation for the second acid. Furthermore, a series of Δ11 monoenoic acids was found in *P.* cf. *lunaecharta*, namely Δ11-20:1, Δ11-22:1, and the unusual Δ11-24:1, Δ11-26:1, and Δ11-28:1. These acids were readily identified *via* their methyl esters and pyrrolidides, whose MS contained the expected molecular ion peaks. The MS of all their pyrrolidides showed a difference of 12 amu between key fragments at *m/z* 224 (C_{10}) and *m/z* 236 (C_{11}), which was clearly indicative of Δ11 unsaturation.

Dienoic fatty acids. Two new dienoic long-chain fatty acids were identified in *P.* cf. *lunaecharta*. The methyl ester of the first acid was eluted just after the well-known Δ5,9-26:2 acid methyl ester (ECL 25.70); its MS had a molecular ion peak at *m/z* 406, suggesting a 26:2 acid structure. The structure was confirmed by the disappearance of the corresponding GC peak upon catalytic hydrogenation and by a molecular ion peak at *m/z* 445 for the corresponding pyrrolidide, as given previously. This acid did not possess typical Δ5,9 unsaturation because it lacked prominent fragmentation ions at *m/z* 81 (for methyl ester) and *m/z* 180 (for pyrrolidide). Both double bonds were readily located by differences of 12 amu between homologous ions at *m/z* 308 (C_{16}) and *m/z* 320 (C_{17}) for the first one, and at *m/z* 362 (C_{20}) and *m/z* 374 (C_{21}) for the second, indicating Δ17 and Δ21 unsaturations, respectively. Thus, the new fatty acid 17,21-hexacosadienoic **[4]** was identified. In the same way, a new octacosadienoic acid was identified because its methyl ester presented an ECL value of 27.66 (eluted after the known Δ5,9-28:2 acid methyl ester) and a molecular ion peak at *m/z* 434; these data suggested a 28:2 acid structure confirmed by the disappearance of the corresponding GC peak upon hydrogenation. The corresponding pyrrolidide displayed a molecular ion peak at *m/z* 473, confirming a 28:2 structure (see above). Differences of 12 amu between homologous ions, first at *m/z* 336 (C_{18}) and *m/z* 348 (C_{19}), and then at *m/z* 390 (C_{22}) and *m/z* 402 (C_{23}), readily indicated Δ19 and Δ23 unsaturations, respectively.

Thus, we identified the new fatty acid 19,23-octacosadienoic [5], which is a homologue of acid [4].

A rare Δ5,9-nonacosadienoic acid, first reported from two Caribbean sponges (12), also was characterized using the same strategy as described previously. The methyl ester was eluted just after 27-methyl-5,9-octacosadienoic acid methyl ester (*iso*-Δ5,9-29:2), recently found in *C.* aff *schulzei* (4). The pyrrolidide of this acid showed a molecular ion peak at *m/z* 487, confirming a 29:2 structure, and a major ion at *m/z* 180 arising from double allylic cleavage between C_7 and C_8, which is typical of Δ5,9 acid pyrrolidides. Methyl branching at the C_{26} position was indicated readily by significant homologous fragmentation ions at *m/z* 431 (protonated C_{25} fragment) and *m/z* 458 (C_{27}), and by the absence of C_{26} fragment at *m/z* 444. Thus, 26-methyl-5,9-octacosadienoic acid (*anteiso*-Δ5,9-29:2) was identified.

Trienoic fatty acids. In our recent report (3), we described the identification of a new Δ5,9 trienoic acid with an n-5 terminal double bond, namely 5,9,25-triacontatrienoic [**8**]. Another new trienoic acid was identified in *P.* cf. *lunaecharta*. Its methyl ester had an ECL value of 25.24, and displayed a molecular ion peak at *m/z* 404 and a major peak at *m/z* 81 typical of Δ5,9 unsaturated acids, suggesting a Δ5,9,n-26:3 structure. These data were consistent with the presence of a molecular ion peak at *m/z* 443 (26:3) and a prominent peak at *m/z* 180 in the MS of its pyrrolidide. A terminal double bond was readily located between the C_{21} and C_{22} atoms because of an interval of 12 amu between the homologous ions at *m/z* 360 (C_{20}) and *m/z* 372 (C_{21}). Thus, the new demospongic acid Δ5,9,21-26:3 [**7**] was characterized.

Three long-chain Δ5,9 trienoic acids also were detected, but the exact position of the third double bond could not be assigned due to the very small amounts available. *Psadaxinella* cf. *lunaecharta* contained a Δ5,9,x-27-3 acid as traces, whereas *T. loeve* contained two minor, different Δ5,9 nonacosatrienoic acids. The latter two isomers were successively eluted as methyl esters (RT, 29.97 min, ECL 28.27; and RT, 30.07 min, ECL 28.30) and showed almost identical MS. Both methyl ester MS displayed the molecular ion peak at *m/z* 446 and the typical fragment at *m/z* 81, which is indicative of a 5,9-nonacosatrienoic structure. Both these peaks disappeared upon catalytic hydrogenation. The corresponding pyrrolidide derivatives were also distinctly eluted (RT, 43.09 and 43.17 min, respectively) and yielded MS that displayed a molecular ion peak at *m/z* 485, indicating a 29:3 structure and an appreciable peak at *m/z* 180 typical of Δ5,9 unsaturation. Both spectra possessed the logical sequence with 14 amu differences until the $C_{20:2}$ fragment at *m/z* 360. Both spectra also possessed this same logical sequence following the ion peak at *m/z* 400 ($C_{23:3}$ fragment). Thus, the third double bond was located between the carbon C_{20} and C_{23} in the two isomeric Δ5,9,y-29:3 and Δ5,9,z-29:3 acids. In the unsaturated fatty acids, all double bonds were of (*Z*) configuration, as the GC/FTIR spectra showed absorptions near 3010 and 700 cm^{-1}, but no absorption near 960 cm^{-1} (17).

DISCUSSION

Of particular interest was the co-occurrence of two phospholipid isoprenoid fatty acids in both sponges, namely 4,8,12-TMTD and phytanic acid (3,7,11,15-TMHD). These fatty acids are frequent in marine environments, but it was previously suggested that these acids do not occur together in the same sponge (18), although they have been found together in the total lipids of *D. fragilis,* in which they are minor components (13). We recently also reported the co-occurrence of the common 4,8,12-TMTD and the not widespread 5,9,13-trimethyltetradecanoic from several marine sponges belonging to the genus *Cinachyrella* (order Spirophorida) originating along the Senegalese coast or in the New Caledonian lagoon (2,4,5). These findings led us to analyze all sponges from there specifically for isoprenoid acids. Thus, we found 4,8,12-TMTD and 3,7,11,15-TMHD together in the Senegalese sponge *P.* cf. *lunaecharta* (order Axinellida, family Axinellidae), which is closely related to *P. lunaecharta,* which also contains both these acids (15). GC/MS single-ion monitoring experiments indicated no trace of 5,9,13-trimethyltetradecanoic acid in these three Axinellida sponges.

The new sponge phospholipid 5-pentacosenoic acid [**2**] identified in *P.* cf. *lunaecharta* has already been found in mycobacteria, as noted by Rezanka (19). We recently reported the first occurrence of 5-octacosenoic acid in phospholipids of *C.* aff *schulzei* (4), and acids Δ5-20:1, Δ5-22:1, Δ5-23:1, and Δ5-26:1 were recently found in freshwater sponges (20). These findings suggest that Δ5 desaturation could arise as a first desaturation step after chain elongation in sponges. It has been demonstrated by incorporation experiments that synthetic 5-hexacosenoic acid is a biosynthetic precursor of 5,9-hexacosadienoic acid (10). The rare fatty acids Δ11-24:1 [**1a**], Δ11-26:1 [**1b**], and Δ11-28:1 [**1c**] found in *P.* cf. *lunaecharta*, in addition to the known Δ11-20:1 and Δ11-22:1, show that a Δ11-desaturase may be operative in this sponge. The acids Δ11-26:1 and Δ11-28:1 were previously found in mycobacteria (19).

Apart from methylene-interrupted polyunsaturated acids, long-chain polyunsaturated acids in sponges almost always possess a Δ5,9 unsaturation pattern. Both new acids Δ17,21-26:2 [**4**] and Δ19,23-28:2 [**5**] represent a new class of long-chain unsaturated fatty acids. Previously, the lower homologous acid Δ11,15-20:2 was identified from a Caribbean sponge (21). As demonstrated by various incorporation experiments, marine sponges are able to synthesize the Δ5,9 demospongic acids by elongation of short-chain saturated or monoenoic fatty acids, followed by desaturation (8–10,22). Thus, the hypothetical Δ21-26:1 and Δ23-28:1 intermediate acids can arise from exogenous 11-hexadecenoic, as previously suggested (23), but subsequent desaturation, such as Δ17 or Δ21 positions, respectively, do not likely occur in a sponge. The lack of Δ19 desaturase activity in a marine sponge was demonstrated (10,22). Furthermore, it is known that the Δ5,9 unsaturation can also be introduced into shorter fatty acids beginning at either the Δ5 or Δ9 position (22).

Thus, myristic acid (14:0) is the shortest acid amenable to chain elongation (22). The Δ9-14:1 acid was described in three marine sponges, which also contained the Δ5,9-18:2 acid (24). The lower homolog Δ5,9-16:2 acid was a major fatty acid of *Chondrilla nucula* (25) and was present in *Chondrosia reniformis* (12). The freshwater sponges *Baicalospongia bacillifera* and *B. intermedia* also contained the Δ5,9-16:2 acid together with the Δ9-14:1, Δ5-16:1, and Δ9-16:1 acids (20).

Therefore, one can likely assume the occurrence of a dienoic short-chain precursor, such as the Δ5,9-14:2 acid, from which the known active homologation enzyme system of the sponges could be operative as follows:

Δ5,9-14:2 → → Δ11,15-20:2 → → Δ17,21-26:2 → Δ19,23-28:2

Also noticeable in *T. loeve* phospholipids was the occurrence of fifteen Δ5,9 long-chain acids accounting for 25.7% of the total mixture, including 5,9,23-triacontatrienoic acid which was the major component (14.8%). Such a large number of Δ5,9-long-chain acids was first observed in *C.* aff *schulzei*, in which they accounted for 30% of the total mixture (4). *Pseudaxinella* cf. *lunaecharta* contained thirteen such acids (30.4%), 5,9-hexacosadienoic acid being the major component (20.3%).

The only known biosynthetic pathway to Δ5,9,n trienoic demospongic acids involves homologation in the n-7 even-chain monoenoic fatty acid series, followed by Δ5 and Δ9 desaturation, as previously demonstrated by incorporation studies in the case of the Δ5,9,19-26:3 from *M. prolifera* (8,10). Palmitoleic acid (Δ9-16:1) incorporated into the sponge *Aplysina fistularis* led to Δ5,9,23-30:3 (9). This sponge was able to homologate short-chain precursors, such as the exogenous acid Δ9-16:1 up to the Δ19-26:1 acid. A further desaturation yielded Δ9,19-26:2, which also occurs in *M. prolifera* (8,10). Such a pathway is involved in the biosynthesis of the relatively frequent Δ5,9,23-30:3 acid (as depicted in Fig. 2A), but the former postulated intermediates, Δ23-30:1 and Δ9,23-30:2 acids (7,23), were not found as natural products in sponge phospholipids before their identification in *T. loeve* (3). Thus, Δ5 desaturation appeared to be a last step in *T. loeve*, but also can occur as a first desaturation in marine sponges.

One of the most interesting results of this work was finding two new trienoic demospongic acids with an n-5 terminal double bond, namely Δ5,9,21-26:3 [7] in *P.* cf. *lunaecharta* and Δ5,9,25-30:3 [8] in *T. loeve*, in addition to the already encountered Δ5,9,23-28:3 acid (4,19,23). In a similar manner as previously mentioned, these trienoic acids most likely arise from short precursors, such as the Δ11-16:1 acid, by chain elongation in the n-5 monoenoic series (23), and by subsequent Δ5,9 desaturation (Fig. 2B). Despite the lack of postulated n-5 monoenoic intermediates in our sponges (acids in brackets), it should be noted that several lower homologs acids have been reported from some sponges, namely Δ11-16:1 (23,26,27), Δ13-18:1 (6), Δ15-20:1 (13), Δ17-22:1 (26), and Δ19-24:1 (21,23,26–28).

In summary, these two Axinellida sponges are of particular interest as they contain several new long-chain unsaturated

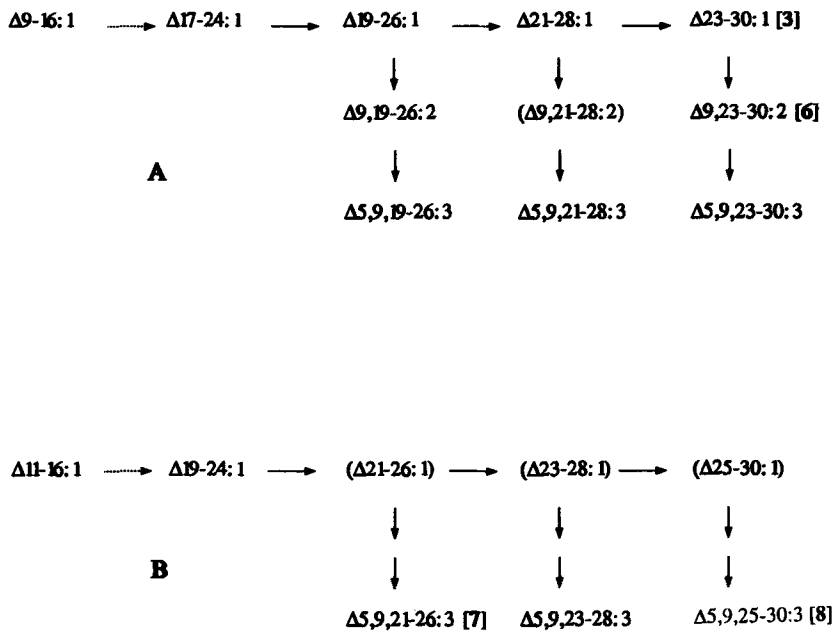


FIG. 2. Proposed biosynthetic pathways for Δ5,9,n demospongic acids with an n-7 (A) or n-5 (B) terminal double bond. All fatty acids were identified in *Trikentrion loeve* or *Pseudaxinella* cf. *lunaecharta* apart from the Δ11-16:1 and Δ19-24:1 acids found in several sponges (see Discussion section) and the Δ9,19-26:2 acid found in *M. prolifera* (Refs. 8,10). Postulated intermediates (in brackets) have not yet been identified as natural products so far.

acids involving unusual biosynthetic possibilities that likely include an n-5 monoenoic chain-elongation step. Nevertheless, much biosynthetic work is needed to demonstrate the origin of these new fatty acids.

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An Unusual Fatty Acid Pattern in *Eranthis* Seed Oil

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ABSTRACT: The current discussion on "renewable resources", and the possibility of gene transfer into rapeseed, has led to many investigations into the biosynthetic pathways leading to industrially useful fatty acids. The various tribes and genera of the plant family *Ranunculaceae* contain a large variety of unusual fatty acids. Seed fatty acid patterns differ considerably from genus to genus and are chemotaxonomically significant indicators. *Eranthis* seed oil has now been found to contain a fatty acid pattern that deviates significantly from the eleven different fatty acid patterns that had been described in this plant family. The main fatty acid (up to 57%) is 13-*cis*,16-*cis*-docosadienoic acid. Other, minor fatty acids found are Δ5-*cis*-monoenoic, -dienoic, and -trienoic fatty acids that had already been reported to be constituents of the genus-specific seed oil fatty acid patterns of various genera from this plant family. Capillary gas–liquid chromatographic retention data indicate that 22:3Δ5*cis*,13*cis*,16*cis* is probably also present. Seed fatty acid chemotaxonomic evidence thus points to a different position of *Eranthis* within the tribes of this plant family. These findings again indicate that the plant family *Ranunculaceae* would be an ideal object to study fatty acid biosynthesis and phylogenetic evolution, because in other genera of this family other types of desaturation and chain elongation mechanisms predominate.
Lipids 31, 201–205 (1996).

The current discussion on renewable resources in the oils and fats field, and the possibility of gene transfer from wild species into agronomically developed crops such as rapeseed, has sparked a large number of investigations into the biosynthetic pathways leading to unusual and industrially useful fatty acids (1, 2). Recent research on seed oils and fatty acids of the plant family *Ranunculaceae* has shown that this plant family contains a large number of different fatty acids, and seed oil fatty acid patterns, that may relate to phylogenetic evolution (3, 4). So far, eleven distinctly different fatty acid patterns have been described in this family (4). The fatty acid composition found in seed oils from mature seeds indicates that in developing seeds of members of this plant family, a variety of desaturation mechanisms and chain elongation mechanisms must be operative that deserve to be studied in more detail (3).

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Abbreviations: ECL, equivalent chain length; GC/MS, gas chromatography/mass spectrometry; GLC, capillary gas–liquid chromatography; RRT, relative retention time.

It seems that in the phylogenetic evolution of the extant members of this plant family from ancient angiospermous predecessors, several steps or mutations must have occurred that influenced or changed enzymes required for fatty acid biosynthesis. Desaturation between carbon atoms 4 and 7, which we had called "front-end"-desaturation in an earlier paper (3), may have changed several times (perhaps by stepwise or independent mutations of the amino acid sequence of the desaturases involved). In some species, chain lengths end at C_{18}, in others at C_{20}. In a number of genera, various combinations exist of "front-end"-desaturation and chain elongation beyond C_{18}. In other genera, both these features were apparently lost during the course of evolution (4). Because of these observations, and provided that the family is indeed monophyletic, the members of this plant family would make an ideal object to study the phylogenetic evolution of seed fatty acid biosynthesis (3,4,5–11).

To test this hypothesis, a number of key species were investigated for seed fatty acid composition where this was previously unknown (4). The findings with *Eranthis hyemalis* are reported in this paper.

EXPERIMENTAL PROCEDURES

Seeds of *E. hyemalis* were kindly provided by Prof. U. Jensen (Department of Botany, University of Bayreuth, Germany) and by M. Payer (St. Pölten, Austria). The seeds were crushed and extracted with hexane as described previously (12). After saponification and removal of the unsaponifiable, fatty acid methyl esters were prepared using the Deutsche Gesellschaft für Fettwissenschaft-BF_3-method (13). Capillary gas chromatography of fatty acid methyl esters and the evaluation of the obtained "fingerprint" patterns was carried out using Hewlett-Packard (Avondale, PA) GLC equipment and a Silar 5 CP column (Chrompack, Middelburg, The Netherlands) as described previously (5,6). To obtain additional fatty acid structural information, the following alternative capillary gas–liquid chromatography (GLC) conditions of different selectivity were used: CP-SIL-88 (Chrompack): 50 m × 0.32 mm; film thickness 0.25 μm; carrier gas He, 1.25 mL/min; temperature program, from 100 to 220°C at 1.5°C/min; 20 min isothermal; Injector and detector at 250°C; split 1:100; 1 μL solution was injected. BPX (SGE, Weiterstadt, Germany): 50 m × 0.22 mm; film thickness 0.25 μm; carrier gas N_2, 0.69 mL/min; temperature program, from 100 to 240°C at 2°C/min;

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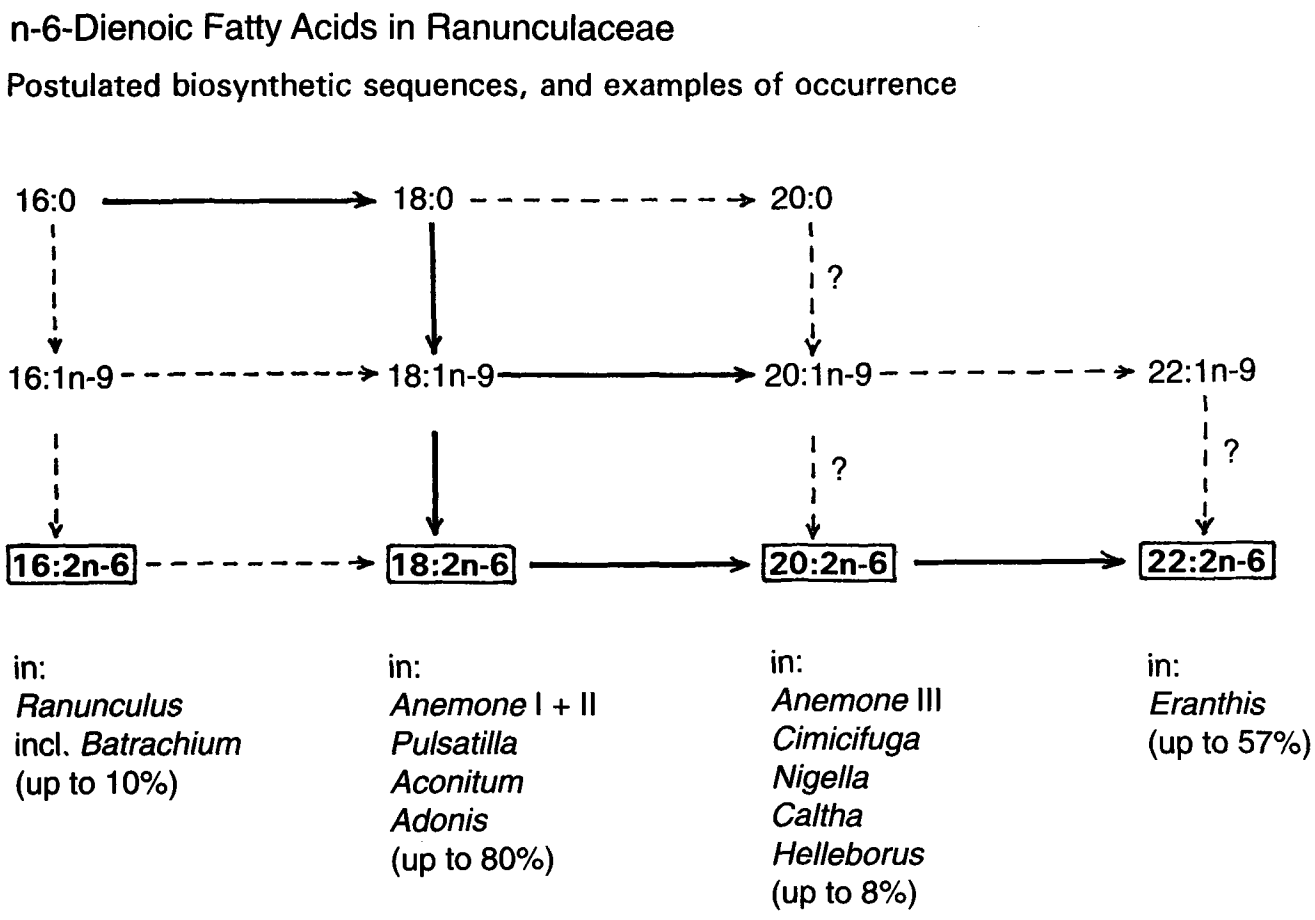
32 min isothermal; injector at 260°C; detector at 240°C; split 1:80; 1 μL solution was injected.

Unusual fatty acids were identified by chromatography on three columns of different polarity and by co-chromatography with standards and samples of known fatty acid composition (6), including 22:2n-6 standards from Nu-Chek-Prep (Elysian, MN) and Larodan (Malmö, Sweden). Dimethyl disulfide adducts of fatty acid methyl esters and fatty acid 4,4-dimethyloxazoline derivatives were prepared and analyzed by gas chromatography/mass spectrometry (GC/MS) as described (14–16).

RESULTS AND DISCUSSION

Several n-6 dienoic fatty acids had been observed before in various genera of the plant family *Ranunculaceae* (Scheme 1). Hexadecadienoic acid (16:2n-6) is a chemotaxonomically significant constituent (at levels of 2–10% of total seed oil fatty acids) of most, if not all, of the species belonging to the genus *Ranunculus* L. incl. subgenus *Batrachium* L. (3,4). Linoleic acid (18:2n-6) is the end product of fatty acid biosynthesis, for example, in "*Anemone* II" (4), and in *Pulsatilla*, *Adonis*, and *Aconitum*, as in many conventional oilseeds (7,8). Eicosadienoic acid (20:2n-6) is found at levels of 7–8% in "*Anemone* III" (3, 4). It is also found, although at lower levels, in *Cimicifuga*, *Helleborus*, *Actaea* and *Caltha* (4). Its presence is highly significant in *Nigella*, where 20:2 is a much larger peak than 20:1 (4).

Seed fatty acid fingerprints (5) show that in the plant family *Ranunculaceae* there are genera where 20:1Δ11*cis* is the most significant peak, while the levels of the corresponding n-6 desaturation product, i.e., 20:2Δ11*cis*,14*cis*, are very low (for example, *Delphinium* and *Consolida*). This ratio is reversed in genera such as *Nigella*, where 20:2 > > 20:1. In still other genera, for example in *Caltha* (9), as well as in *Anemone* III (4) and *Cimicifuga* (Aitzetmüller, unpublished data), 20:2 is apparently further desaturated at Δ5, i.e., at the "front end" closer to the COOH group, to yield 20:3Δ5*cis*, 11*cis*,14*cis*, which is a major fatty acid in *Caltha* (3, 4, 9). Other C_{16}, C_{18}, and C_{20} fatty acids—with the highly significant exception of 18:2n-6—are apparently also desaturated at Δ5 in a number of genera, for example in *Caltha*, *Cimicifuga*, *Actaea* and *Anemone* III (4), leading to a whole range of various Δ5*cis* fatty acids. In view of this, it is rather surprising that an 18:3 polyenoic fatty acid with a *cis* double bond in position Δ5 has not yet been observed in this plant family. However, the corresponding *trans*-fatty acid, 18:3Δ5*trans*,9*cis*, 12*cis*, is prominent in *Aquilegia* and *Thalictrum* (10,11) and



SCHEME 1

may indeed be a chemotaxonomic marker (3,4) of the subfamily *Thalictroideae* (14) or *Isopyroideae* (15). The fatty acid 18:4Δ5*trans*,9*cis*,12*cis*,15*cis* has recently been tentatively identified as a minor acid in *Isopyrum* (4), and 18:4Δ5*cis*,9*cis*,12*cis*,15*cis* is apparently a minor acid present in both *Caltha* and *Cimicifuga* (4).

Fatty acid methyl esters obtained from *E. hyemalis* seed oil gave a totally different fatty acid pattern in capillary GLC (Fig. 1). The main fatty acid (up to 57%) was 22:2n-6 (or 22:2Δ13*cis*,16*cis*). This was identified *via* its relative retention times (RRT) and equivalent chain length (ECL) values on three different gas chromatographic columns of different selectivity (6), including direct comparison and co-chromatography with suitable reference standards and other seed oils where it occurs (5). The observed ECL value for the 22:2n-6 standard on Silar 5CP was 22.86, and the same ECL value was found for the major *Eranthis* fatty acid. Likewise the ECL values on CP-SIL-88 and BPX were 23.32 and 23.00, respectively. Co-chromatography of *Eranthis* methyl esters with authentic 22:2n-6 yielded only one peak in the 22:2n-6 region of the chromatogram. GC/MS of the methyl ester gave the molecular ion of 350 as expected for 22:2. GC/MS of the dimethyl disulfide adduct indicated fragmentation compatible with a 22:2n-6 structure (major diagnostic fragments at *m/e* 265 and 155). GC/MS of the 4,4-dimethyloxazoline derivative also confirmed the 22:2n-6 structure (major diagnostic fragments at m/e 252, 264, 292, and 304).

However, *Eranthis* apparently also contained a whole range of Δ5*cis* desaturation products as minor seed oil fatty acids (Table 1). Most of these were identified by comparison with *Caltha*, *Actaea*, and *Cimicifuga* (4) fatty acids on three GLC columns of different selectivity. They were identical with those fatty acids whose presence had already been established in *Caltha* (9). Most significantly, and again as in *Caltha*, *Actaea*, *Cimicifuga*, and "*Anemone* III" (4), the Δ5*cis*-desaturation product of linoleic acid (i.e., pinolenic acid, 18:3Δ5*cis*,9*cis*,12*cis*) was missing also in the *Eranthis* seed oil fatty acid pattern. Apart from these fatty acids, *Eranthis* apparently also contained the Δ5*cis* desaturation product of 22:2n-6, i.e., the fatty acid 22:3Δ5*cis*,13*cis*,16*cis*—a fatty acid hitherto not yet described and a homologue of the main fatty acid of *Caltha* (9). This identification, however, is based solely on GLC column selectivity, RRT and ECL considerations (17,18) and therefore should be considered as tentative—no reference material for co-chromatography was available. The same applies to another noticeable peak, which is

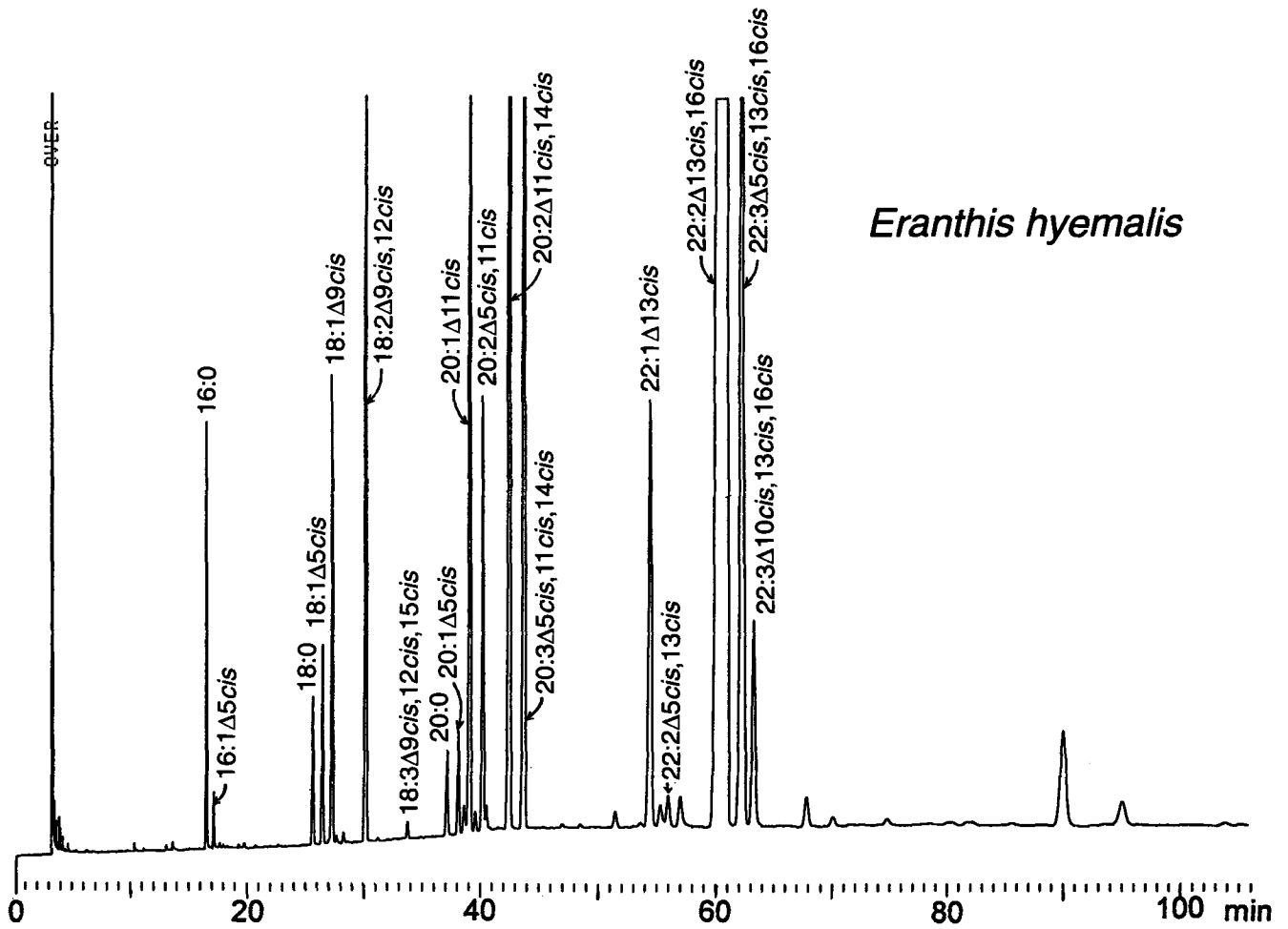


FIG. 1. Capillary gas chromatogram of fatty acid methyl esters obtained from *Eranthis hyemalis* seed oil.

TABLE 1
Fatty Acid Composition of *Eranthis hyemalis* Seed Oils

Lipid in seed (wt%)	GLC (area %)	
Fatty acids	30.1[a]	32.9[b]
14:0	trace	trace
15:0	trace	trace
16:0	1.2	0.6
16:1Δ5*cis*	0.2	0.1
18:0	0.6	0.5
18:1Δ5*cis*	0.8	0.6
18:1Δ9*cis*	1.8	1.5
18:1Δ11*cis*	trace	trace
18:2Δ9*cis*,12*cis*	3.7	2.3
18:3Δ9*cis*,12*cis*,15*cis*	0.1	0.1
20:0	0.4	0.5
20:1Δ5*cis*	0.5	0.4
20:1Δ11*cis*	3.9	4.8
20:2Δ5*cis*,11*cis*[c]	1.9	1.9
20:2Δ11*cis*,14*cis*	6.5	6.9
20:3Δ5*cis*,11*cis*,14*cis*	5.8	5.8
22:0	0.1	0.1
22:1Δ13*cis*	3.3	3.9
22:2Δ5*cis*,13*cis*[c]	0.3	0.3
22:2Δ13*cis*,16*cis*	55.7	57.0
22:3Δ5*cis*,13*cis*,16*cis*[c]	8.1	7.8
22:3Δ10*cis*,13*cis*,16*cis*[c]	1.7	1.6
Others	3.4	3.3

[a]German sample.
[b]Austrian sample.
[c]Identification tentative [by gas–liquid chromatography (GLC)].

most likely 20:2Δ5*cis*,11*cis*—a fatty acid so far found only in low amounts in other *Ranunculaceae*. This too was identified by extrapolation of the GLC (RRT and ECL) data of other Δ5 acids in the *Ranunculaceae*, as well as by a direct comparison with the equivalent minor fatty acid peaks present in *Caltha* (3,5,6,9,19). *Eranthis* seeds obtained from two different sources in Germany and in Austria showed essentially the same fatty acid pattern (Table 1).

The results described here support the position of *Eranthis* as a separate tribe or subtribe within the *Ranunculaceae* (20, 21). Moreover, Jensen (22) had observed a serologically closer relationship between *Eranthis* and the *Actaea–Cimicifuga* group. This, too, is supported by the present results, which indicate the presence of a similar set of Δ5 fatty acids in *Eranthis*, compared with those found in *Actaea* and *Cimicifuga* (cf. Table 3 in Ref. 4).

Apparently, in *Eranthis* still another major fatty acid biosynthetic pathway, different from those postulated before in other *Ranunculaceae* genera (3) is operative. Fatty acid biosynthesis in *Eranthis* does not stop at 20:1 (as in *Consolida*) or at 20:2 (as in *Nigella*), and it is not deviated to the main production of 20:3Δ5*cis*,11*cis*,14*cis* [as in *Caltha* (Ref. 9)]. Also, Δ5*trans* fatty acids (10,11,19) as in the subfamily *Isopyroideae* (20) were not observed. Instead, apparently further chain elongation occurs and leads to the accumulation of 22:2Δ13*cis*,16*cis* as the major end product of fatty acid biosynthesis (up to 57%). Presumably, 20:2Δ11*cis*,14*cis* is the predecessor [4]. However, a whole range of Δ5*cis* fatty acids—similar to those found in *Caltha*, *Cimicifuga*, and *Actaea*—is also present in *Eranthis* as minor fatty acids, accompanying the predominant 22:2n-6 (Table 1).

The phylogenetic relationships between members of the family *Ranunculaceae* have recently been the subject of much discussion (cf. Refs. 20,21,23, and references quoted therein). If the *Ranunculaceae* are indeed monophyletic, the findings on *Eranthis* presented here again support the view (3, 4) that the members of this plant family are ideal study objects for the phylogenetic evolution of seed fatty acid biosynthesis and the enzymes involved in this. It would certainly be interesting to find out more about the activities and specificities, and the amino acid sequences, of the various desaturases and elongases required to produce all these different unusual fatty acids. The presence of these enzymes in the seeds may have contributed significantly to the earlier serological findings of Jensen (22) when, in contrast to his later work, antibodies against total seed proteins were used. During the evolution of this plant family, stepwise changes in the primary amino acid sequences of the enzymes involved here may have occurred by way of mutation. From a better understanding of these evolutionary developments, important conclusions could be drawn with regard to genetic engineering of enzymes that may be involved in the biosynthesis of industrially useful fatty acids with unusual double bond positions. Eventually, such work may lead to new and improved technical oil crops as renewable resources (1,2,24), for future decades when petroleum resources are depleted.

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Stereospecific Analysis of Triacylglycerols Rich in Long-Chain Polyunsaturated Fatty Acids

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ABSTRACT: Six oils of marine, algal, and microbial origin were analyzed for stereospecific distribution of component fatty acids. The general procedure involved preparation of *sn*-1,2-(2,3)-diacylglycerols by partial deacylation with ethylmagnesium bromide or pancreatic lipase, separation of X-1,3- and *sn*-1,2(2,3)-diacylglycerols by borate thin-layer chromatography, resolution of the *sn*-1,2- and *sn*-2,3-enantiomers by chiral phase high-performance liquid chromatography following preparation of dinitrophenylurethane derivatives, and determination of the fatty acid composition by gas chromatography. Unexpected complications arose during a stereospecific analysis of triacylglycerols containing over 33% of either 20:4 or 22:6 fatty acids. The *sn*-1,2(2,3)-diacylglycerols made up of two long-chain polyunsaturated acids migrated with the X-1,3-diacylglycerols and required separate chiral phase resolution. Furthermore, the enzymatic method yielded *sn*-1,2(2,3)-diacylglycerols, overrepresenting the polyenoic species due to their relative resistance to lipolysis, but prolonged digestion yielded correct composition for the 2-monoacylglycerols. The final positional distribution of the fatty acids was established by pooling and normalizing the data from subfractions obtained by normal- and chiral-phase separation of diacylglycerols. The molecular species of X-1,3-, *sn*-1,2- and *sn*-2,3-diacylglycerol dinitrophenylurethanes were identified by chiral-phase liquid chromatography/mass spectrometry with electrospray ionization, which demonstrated a preferential association of the paired long-chain acids with the *sn*-1,2- and *sn*-2,3-diacylglycerol isomers.

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Knowledge of the stereospecific distribution of fatty acids in natural fats is of interest because of the positional specificity of the lipolytic enzymes (1–3), the intestinal absorption, and reesterification of intact 2-monoacylglycerols (MAG) (4,5), and the desirability to reproduce the structure of certain scarce natural fats and oils (6,7) by blends of natural or synthetic fats and oils. Recently specialty or designer oils rich in n-6 and/or n-3 polyunsaturated C_{20} and C_{22} fatty acids have been introduced for dietary supplementation (8), but the triacylglycerol (TAG) structure of these oils has not been determined. In fact, application of conventional methods of stereospecific analyses to such oils has proved difficult due to isolation of nonrepresentative diacylglycerol (DAG) intermediates (Myher, J.J., Kuksis, A., and Park, P.W., 1994, unpublished results). We have traced this problem to the overlap of long-chain *sn*-1,2(2,3)-DAG with short- and medium-chain X-1,3-DAG during the boric acid thin-layer chromatography (TLC) used for the preliminary resolution of the *sn*-1,2(2,3)- and X-1,3-DAG. Usually natural oils contain only one long-chain polyunsaturated fatty acid per TAG molecule and borate TLC yields representative *sn*-1,2(2,3)-DAG from Grignard degradation (5,9). The present study demonstrates that valid stereospecific analyses of the oils enriched in polyunsaturated fatty acids can be obtained provided special care is taken in the isolation and identification of the enantiomeric DAG intermediates.

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Abbreviations: DAG, diacylglycerol; DNPU, dinitrophenylurethane; ECN, equivalent carbon number; ELSD, evaporative light-scattering detection; GC, gas chromatography; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; MAG, monoacylglycerols; PN, partition number; TAG, triacylglycerol; TLC, thin-layer chromatography.

EXPERIMENTAL PROCEDURES

Materials. Six polyunsaturated oils of marine (tuna) (B), microalgal (*Chrypthecodinium cohnii*) (C and D) and microbial micelia (*Mortierella alpina*) (A, E, and F) origin were available in the laboratory. Three were rich in arachidonic acid (A, E, and F), and three were rich in docosahexaenoic acid (B, C, and D). Reference *sn*-1,2- and *sn*-2,3-DAG dinitrophenylurethane (DNPU) derivatives prepared from standard TAG were available in the laboratory from previous studies (10,11). The 3,5-dinitrophenylisocyanate was purchased from Sumitomo Chemical Co. (Osaka, Japan). Ethylmagnesium bromide and boric acid were obtained from Aldrich Chemical Co. (Milwaukee, WI). Pancreatic lipase was obtained from Sigma Chemical Co. (St. Louis, MO).

Purification of TAG. Five drops of each oil were dissolved in 1 mL of chloroform, and 250 μL of the solution was applied to a 20 × 20 cm TLC plate coated with a 250-μm thin-layer of Silica Gel H (Merck, Darmstadt, Germany). The plate was developed with hexane/ethyl acetate (88:11, vol/vol) (12). The TLC bands corresponding to standard steryl ester, TAG, and phosphatidylcholine were scraped off and eluted

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with chloroform/methanol (2:1, vol/vol) and the solvents evaporated under nitrogen. Care was taken in collecting the TAG fraction to avoid loss of short- and long-chain TAG that might have trailed or preceded the bulk of species. The TAG fraction was subjected to immediate stereospecific analysis, while the other fractions were saved for later analyses. Aliquots were taken for nonpolar capillary gas chromatography (GC) of fatty acids and reversed-phase high-performance liquid chromatography (HPLC) of the intact lipid esters (see below).

Partial deacylation of TAG. Ten mg of the purified TAG were dissolved in dry diethyl ether (1 mL), freshly diluted 0.5 M ethyl magnesium bromide in dry ethyl ether (250 μL) was added, and the mixture was shaken for 1 min before glacial acetic acid (6 μL) in hexane (5 mL) and water (2 mL) were added to stop the reaction (5). The organic layer was washed twice with water (2 mL) and dried over anhydrous sodium sulfate. After evaporating the solvent in a stream of nitrogen at room temperature, the mixture of deacylation products was separated by borate TLC (13) using chloroform/acetone (88:12, vol/vol). The *sn*-1,2(2,3)- and X-1,3-DAG were separately recovered. Alternatively, the TAG were partially deacylated by digestion with pancreatic lipase using another established procedure (14). The digestion was performed in presence of gum arabic for 30 s and the digestion products extracted with diethyl ether. The *sn*-1,2(2,3)- and X-1,3-DAG and 2-MAG, were resolved by boric acid TLC and separately recovered as described above. For preparation of representative 2-MAG the digestion was prolonged to 2–3 min. After taking aliquots of the DAG fractions for fatty acid analyses, the remainder of the X-1,3- and *sn*-1,2(2,3)-DAG were converted into the DNPU derivatives for chiral phase HPLC resolution (see below).

Preparation of 3,5-DNPU. The X-1,3- and the *sn*-1,2(2,3)-DAG (1 mg) were dissolved in dry toluene (400 μL) containing 40 μL of dry pyridine and about 2 mg of 3,5-dinitrophenylisocyanate and allowed to stand for 1 h at room temperature (5). The partly overlapping long-chain *sn*-1,2(2,3)-DAG and the short-chain X-1,3-DAG were converted to the DNPU derivatives together, while the short-chain *sn*-1,2(2,3)-DAG and the long-chain X-1,3-DAG were derivatized separately. The resulting 3,5-DNPU derivatives were purified by TLC on a silica gel plate (Kodak, Rochester, NY). Prior to use, the plate was activated at 110–120°C for 1 h. The reaction mixture, dissolved in 400 μL of chloroform, was spotted on the plate and developed up to 15 cm using petroleum ether/1,2-dichloroethane/ethanol (40:10:3, by vol) as the developing solvent. Bands were located under ultraviolet and the 3,5-DNPU fractions (R_f = 0.5–0.6) were scraped off the plate. The pure 3,5-DNPU were recovered from the adsorbent by extraction with diethyl ether.

Chiral phase HPLC. The enantiomeric *sn*-1,2- and *sn*-2,3-DAG in the *sn*-1,2(2,3)- and in the X-1,3-DAG fractions were resolved using the chiral (R)-(+)-1-(1-naphthyl)ethyl-amine phase bonded to silica (YMC Pack A-KO_3; YMC Inc., Kyoto, Japan) and installed in a Hewlett-Packard (Palo Alto, CA) Model 1090 liquid chromatograph, as previously described (10,11). The analysis was performed isocratically at 23°C using hexane/1,2-dichloroethane/ethanol (40:10:1, by vol) as the mobile phase at a constant flow rate of 0.5 mL/min. Peaks were monitored at 254 nm using a variable wavelength detector. The corresponding *sn*-1,2- and *sn*-2,3-DAG from the two separations were collected and combined for fatty acid analysis.

GC of fatty acids. The fatty acid composition of the purified TAG and their deacylation products was determined following transmethylation with 6% H_2SO_4-MeOH as described (12). The methyl esters were resolved by polar capillary GC and the component acids were quantitated by flame-ionization detection (12).

HPLC of TAG. Intact TAG were resolved by reversed-phase HPLC as previously described (15). The eluted peaks were monitored and quantitated by an evaporative light-scattering detector (ELSD).

Liquid chromatography/mass spectrometry (LC/MS). LC/MS with on-line electrospray of the DAG DNPU was performed using the YMC Pack A-KO_3 column installed in a Hewlett-Packard Model 1090 liquid chromatograph. The separations of X-1,3-, *sn*-1,2- and *sn*-2,3-DAG were obtained with hexane/dichloromethane/ethanol (40:10:1). After post-column mixing with 0.3 mL/min isopropanol/1% aqueous ammonia, mass spectra of eluting peaks were acquired by negative mode nebulizer assisted electrospray interfaced with a Hewlett-Packard MS Engine Quadrupole mass spectrometer (16).

Calculations. The mole % fatty acid composition of the *sn*-1, *sn*-2 and *sn*-3 positions of the TAG molecules were calculated by subtraction of the mole % fatty acid compositions as follows (5):

$$sn\text{-}2 = (\text{2-MAG}), \text{ from pancreatic lipase deacylation} \quad [1]$$

$$sn\text{-}1 = 2 \times (sn\text{-}1,2\text{-DAG}) - (sn\text{-}2\text{-MAG}) \quad [2]$$

$$sn\text{-}3 = 2 \times (sn\text{-}2,3\text{-DAG}) - (sn\text{-}2\text{-MAG}) \quad [3]$$

Alternatively, the positional fatty acid distribution was calculated as follows:

$$sn\text{-}1 = 3 \times (\text{total TAG}) - 2 \times (sn\text{-}2,3\text{-DAG}) \quad [4]$$

$$sn\text{-}3 = 3 \times (\text{total TAG}) - 2 \times (sn\text{-}1,2\text{-DAG}) \quad [5]$$

The final composition of the *sn*-1 and *sn*-3 positions was obtained by averaging the results of the two calculations.

RESULTS AND DISCUSSION

Preliminary characterization. TLC of the oil samples in a neutral solvent system revealed the presence of a large TAG band preceded by a faint band corresponding to steryl esters and small amounts of polar material left at the origin of the plate. There was evidence for the presence of trace amounts of free sterols or free DAG in the E and F, and C and D oil samples. Table 1 gives the fatty acid composition of the purified TAG

TABLE 1
Fatty Acid Composition of Purified Triacylglycerols (mole %)

Fatty acids	Triacylglycerols					
	A	B	C	D	E	F
10:0			0.18	0.75		
12:0	0.00	0.04	3.42	6.47		
14:0	0.36	3.70	14.79	19.76	0.38	0.35
15:0	0.17	1.29	0.06	0.01	0.12	0.14
16:0	13.93	22.29	15.31	17.08	8.01	9.31
16:1n-7	0.21	5.04	1.59	1.76	0.08	0.08
17:0	0.36	2.31	0.08	0.01	0.46	0.54
18:0	6.43	5.77	1.68	0.24	11.18	10.91
18:1n-9	15.53	15.48	14.63	9.01	14.80	8.13
18:2n-6	21.52	1.58	1.41	0.41	6.88	5.68
18:3n-6	3.17	0.14	0.41		2.93	3.25
20:0	0.65	0.43	0.18	0.03	1.06	1.12
18:3n-3	2.13	0.47	0.13	0.14	0.23	0.03
20:1	0.53	1.07	0.14	0.06	0.42	0.50
20:2	0.44	0.30	0.15	0.02	0.61	0.74
20:3n-6	3.10	0.13	0.31	0.01	2.11	2.68
22:0	1.84	0.25	0.32	0.02	0.00	0.00
20:4n-6	24.75	2.12	6.45	0.08	47.87	52.98
22:1n-9	0.09	0.13	0.00			
20:5n-3	0.00	7.10	0.06	0.05	0.22	0.26
24:0	2.89		0.19	0.01	1.03	1.48
24:1		0.42	0.08			
22:6n-3	0.02	29.89	37.21	43.51	0.05	0.04
Other	1.88	0.02	1.22	0.57	1.56	1.78

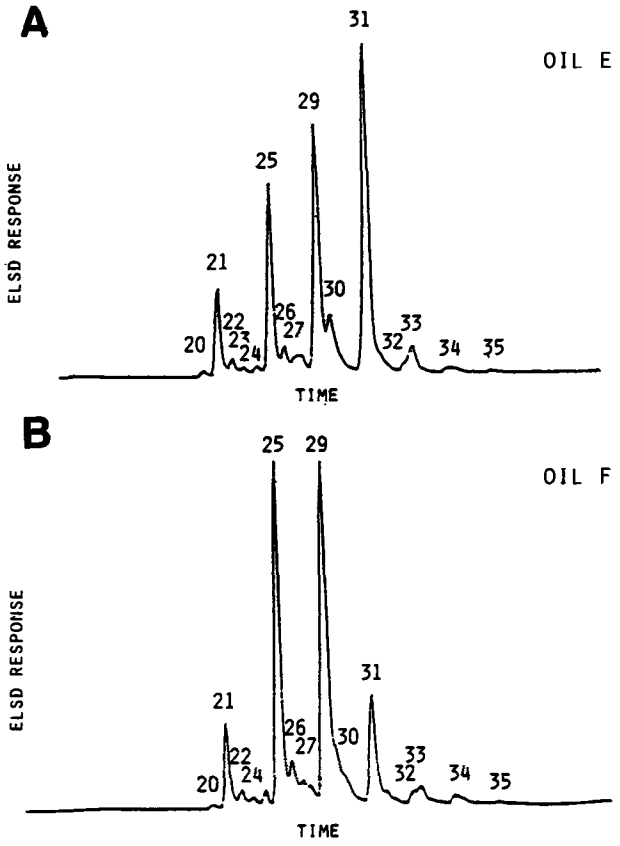


FIG. 1. Reversed-phase high-performance liquid chromatography (HPLC) profiles of E and F polyunsaturated oils as obtained with light-scattering detector. HPLC conditions: Hewlett-Packard Model 1050 liquid chromatograph (Palo Alto, CA) equipped with a Supelcosil LC-18 column (25 × 0.46 cm. i.d.) coupled to a Varex evaporative light-scattering detection (ELSD) II light scattering detector. Solvent: linear gradient of 10–90% isopropanol in acetonitrile at 25°C over a period of 70 min (1 mL/min). Peak identification by fractional equivalent chain-length number is given in Table 2.

fractions. It is seen that the TAG samples can be arranged in three groups on the basis of the major fatty acid content. Thus, samples E and F are characterized by a high content of 20:4, which averages about 50% of total. In contrast, samples C and D are characterized by a high content of 22:6, which averages about 40%. The C sample also contained a significant amount of 20:4 (6.5%). Finally, samples A and B contain mixed polyunsaturates as major components. Thus, sample A contains both 18:2 (21%) and 20:4 (25%) as polyunsaturates, while sample B contained 20:5 (7.1%) and 22:6 (29.9%) as the polyunsaturates. Sample B also contained 16:1 (5.0%) and was by far the most complicated of all the oil samples.

Figures 1–3 show the reversed-phase HPLC elution profiles of the purified TAG as monitored by the ELSD. Sample C oil gives excellent separation with almost complete baseline resolution (often complete resolution), despite the high content of 22:6 in these samples. The resolution of the E sample is poorer, and poor resolution appears to be due mainly to the higher content and greater variety of the unsaturated acids. Less than perfect separation was obtained also for the other 20:4-rich TAG oil, sample A. There was an abundance of double peaks. Likewise, double peaking was present in the B sample, although extensive resolution was still obtained.

Tables 2 and 3 give the quantitative composition of the TAG peaks by equivalent carbon number (ECN) as estimated by HPLC with ELSD. The reversed-phase HPLC resolves TAG by partition or ECN, where one double bond decreases the retention time equivalent to shortening the carbon chain by two methylene units. However, fractional ECN numbers also may be calculated to accommodate slight differences in retention time due to differences in location of the double bond in the fatty chain and of the fatty acid in the TAG molecule. In the present instance, the ECN values range from 38 to 54. Although a tentative identification of the major TAG species can be calculated from statistical consideration of the composition and positional distribution of the fatty acids, exact identification requires MS (see below). Thus, sample D would be expected to contain 14:0-22:6-22:6 (58:12 = PN 34), 16:0-22:6-22:6 (60:12 = PN 36) and 14:0-16:0-22:6 (52:6 = PN 40), where PN is the partition number (the acyl carbon number minus two times the number of double bonds), and major peaks can be found corresponding to these structures in the elution pattern. The identity of the major species can be confirmed and the identity of others established by on-line MS, which, however, was not completed on all samples as it was not considered necessary for stereospecific analysis of the positional distribution of the fatty acids.

Deacylation of TAG. Accurate stereospecific analysis depends on the precise execution of each one of the multistep

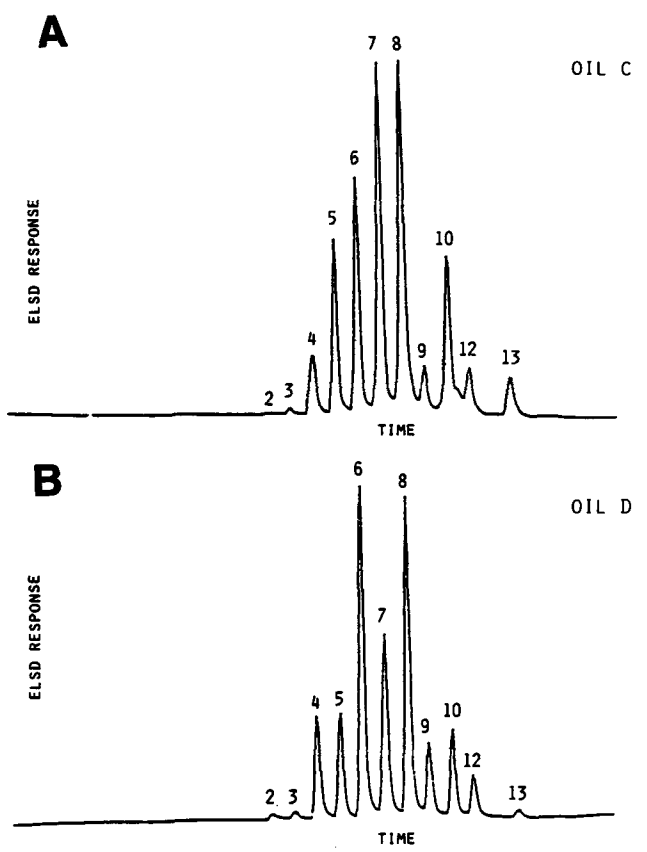


FIG. 2. Reversed-phase HPLC profiles of C and D polyunsaturated oils as obtained by light-scattering detector. HPLC conditions as given in Figure 1. Peak identification as given in Table 2. See Figure 1 for abbreviations.

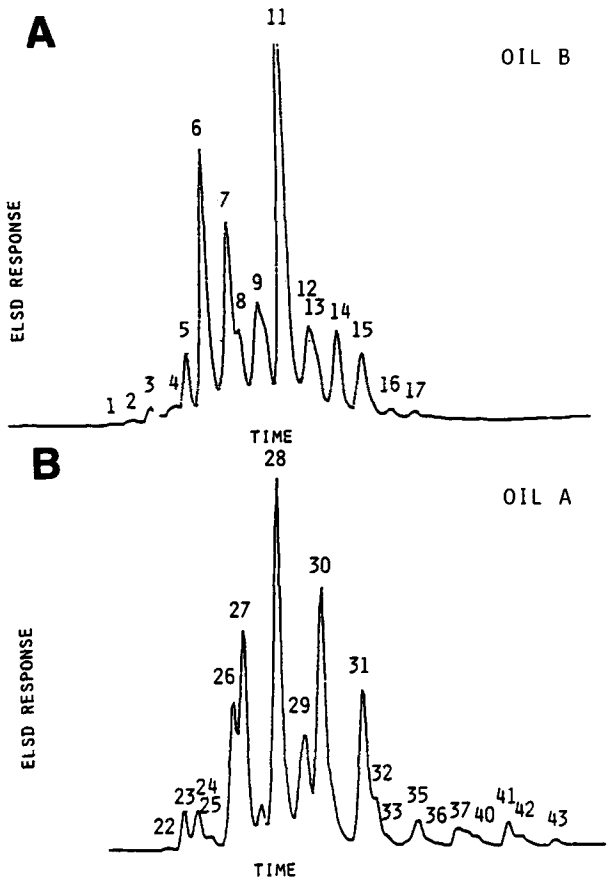


FIG. 3. Reversed-phase HPLC profiles of B and A polyunsaturated oils as obtained with light-scattering detector. HPLC conditions and abbreviations as given in Figure 1. Peak identification as given in Table 3.

procedure and frequent reconstitutions must be made to ascertain that each step has been adequately completed. Of critical importance is the formation of representative (random) *sn*-1,2(2,3)-DAG. In this study, neither the Grignard reaction nor pancreatic lipase yielded representative *sn*-1,2(2,3)-DAG, as isolated by borate TLC. This was due to the faster TLC migration of the long-chain polyunsaturated *sn*-1,2(2,3)-DAG, which led to an overlap with the X-1,3-DAG. Only after chiral-phase HPLC of the X-1,3-DAG was it recognized that the long-chain polyunsaturates in this fraction were due to contamination with *sn*-1,2(2,3)-DAG (see below). Because of the chain-length segregation and background contamination, it was also difficult to obtain representative samples from the small 2-MAG fraction produced during the Grignard reaction. However, representative 2-MAG could be recovered by prolonged pancreatic lipolysis, which yields 2-MAG as the final reaction products. In this case the TLC band was so strong that a representative 2-MAG fraction was readily obtained without contamination and undesirable segregation based on chain length. Pancreatic lipase is known to discriminate against the long-chain polyunsaturated fatty acids during brief digestion periods leading to nonrepresentative formation of the DAG intermediates. Table 4 shows the fatty acid composition of the original and reconstituted TAG for the D oil, along with corrected and calculated composition of the *sn*-1,2(2,3)-DAG obtained by Grignard deacylation, and of 2-MAG recovered from pancreatic and Grignard deacylation. There is reasonably good agreement between the corrected experimental *sn*-1,2(2,3)-DAG and the *sn*-1,2(2,3)-DAG calculated from the original TAG and the 2-MAG derived from pancreatic digestion. Thus, it became necessary to correct the *sn*-1,2- and *sn*-2,3-DAG compositions for loss of the polyunsaturated species in the X-1,3-DAG fraction in all the oils enriched in polyunsaturated long-chain acids. The need for such corrections was minimal in case of the A and B oils, the *sn*-1,2(2,3)-DAG of which showed only limited overlap with the X-1,3-DAG.

Stereospecific analyses. Figure 4 shows the chiral-phase HPLC separation of the *sn*-1,2- and *sn*-2,3-DAG derived from two of the TAG samples, one rich in 20:4 (F) and the other rich in 22:6 (D) fatty acid. Both show excellent resolution of the *sn*-1,2- and *sn*-2,3-enantiomers, with the latter emerging last. The X-1,3-isomers not removed by borate TLC emerge just ahead of the *sn*-1,2-enantiomers. There is considerable resolution of molecular species within the *sn*-1,2- and *sn*-2,3-enantiomers. The species were identified by chiral-phase LC/MS with electrospray. As noted above, during borate TLC, the long-chain *sn*-1,2(2,3)-DAG were observed to mi-

TABLE 2
Relative Proportions of Triacylglycerol ECN Values in the Test Oils as Estimated by High-Performance Liquid Chromatography with Evaporative Light-Scattering Detection (area %)[a]

Peak no.	Fractional ECN	Triacylglycerol oils C	D	E	F
1	35.76	trace	trace		
2	37.02	1.18	1.56		
3	37.97	1.50	1.96		
4	38.88	6.03	9.18		
5	39.86	10.34	9.32		
6	40.79	13.37	19.93		
7	41.77	17.64	14.50		
8	42.69	18.95	19.59		
9	43.59	5.44	8.06		
10	44.61	11.01	8.67		
11	44.99	3.4	0.0		
12	45.47	5.27	5.51		
13	47.37	4.49	1.72		
14	50.40	1.37			
20	40.232			1.32	1.01
21	40.854			7.47	7.33
22	41.408			2.74	2.84
23	41.848			1.93	2.21
24	42.437			1.72	2.57
25	42.986			13.25	20.25
26	43.545			3.92	5.38
27	44.018			2.65	3.35
28	44.231			3.14	3.26
29	44.942			18.97	23.87
30	45.523			8.48	8.62
31	47.098			22.90	10.64
31a	47.615			3.07	
32	48.646			2.45	2.95
33	49.020				
34	50.549			4.77	2.63
35	52.450			1.90	
Other		0.01		0.68	3.09

[a]Abbreviation: ECN, equivalent carbon number.

TABLE 3
Relative Proportions of Triacylglycerol Species by ECN in the Test Oils as Estimated by High-Performance Liquid Chromatography with Evaporative Light-Scattering Detection (area %)[a]

Peak no.	Fractional ECN	Triacylglycerol oils A	B
1	38.388		
2	39.175		1.56
3	39.810		2.65
4	40.619		2.87
5	41.072		5.17
6	41.690		13.59
7	42.548		11.74
8	43.392		5.62
9	43.566		11.30
10	43.778		
11	44.447		19.27
12	45.366		9.75
13	45.592		
14	46.334		7.13
15	47.197		5.10
16	48.167		2.39
17	49.060		1.98
22	40.221	0.5	
23	40.851	2.1	
24	41.331	2.9	
25	41.820	1.5	
26	43.273	5.7	
27	42.938	8.4	
27a	43.576	2.6	
28	44.097	12.7	
29	45.066	6.8	
30	45.633	35.6	
31	47.093	7.4	
32	47.521	2.6	
33	47.951	1.2	
34	48.615	0.0	
35	49.032	3.0	
36	49.465		
37	50.422	2.8	
38	50.787		
39	51.195		
40	51.604		
41	52.349	2.0	
42	52.835	1.2	
43	54.148	1.0	

[a]See Table 2 for abbreviation.

grate with the short-chain X-1,3-DAG. Figure 5 shows the chiral-phase LC/MS elution patterns for the selected ions representing the mass of the 22:6-22:6 species. Peaks of comparable intensity are seen to be eluted in the *sn*-1,2- and *sn*-2,3-enantiomer regions of the chiral chromatogram. Similar separations were necessary for the oils rich in 20:4 acid. In this instance, the *sn*-1,2-enantiomers would be expected to show higher intensity than the *sn*-2,3-enantiomers.

Tables 5–7 give the corrected fatty acid composition of the *sn*-1, *sn*-2, and *sn*-3 positions of the six oils. It is seen that the 20:4 in E and F (Table 5) is found in the *sn*-1 position in double the concentration in the *sn*-3 position, with an intermediate value in the *sn*-2 position. The *sn*-3 position contains the bulk of 16:0 and 18:0, while the 18:1 and 18:2 are largely found in the *sn*-2 position. In contrast the 22:6 in C and D (Table 6) was found mainly in the *sn*-2 position, with the *sn*-1 position containing about half as much and the *sn*-3 position an intermediate value of this acid (especially in D). While 18:1 again was preferred for the *sn*-2 position, the saturated fatty acids 14:0 and 16:0 were about equally divided between the *sn*-1 and *sn*-3 positions with much less found in the *sn*-2 position. The 22:6 distribution in B (Table 7) again was confined largely to the *sn*-2 and *sn*-3 positions, with about one quarter of each in the *sn*-1 position. Interestingly, in this instance the 18:1 was divided nearly equally between the *sn*-1 and *sn*-3 positions, with much smaller amounts in the *sn*-2 position. The saturated acids, 14:0 and 16:0, were found primarily in the *sn*-1 and *sn*-2 positions, while 18:0 was found primarily in the *sn*-1 position. The 20:4 distribution in A (Table 7) was characterized by nearly equal proportions of the acid in all three positions of the TG, while the other major unsaturated acids, 18:1 and 18:2, were found in the *sn*-2 position in approximately twice the proportions found in *sn*-1 and *sn*-3 positions, which were about equal. The 16:0 was found in high proportions in *sn*-1 and *sn*-3 positions, while 18:0 was

TABLE 4
Fatty Acid Composition of Original and Reconstituted TAG[a] of Sample D, the Corrected and Calculated[b] Grignard *sn*-1,2(2,3)-DAG, and Pancreatic Lipase 2-MAG[c]

Fatty acids	TAG		2-MAG		1,2(2,3-DAG)	
	Original	Reconstituted (G)	Grignard	Lipase	Grignard	Calculated
10:0	0.75	0.75	0.00	0.00	0.00	0.56
12:0	6.47	6.47	0.53	0.44	1.78	4.97
14:0	19.76	19.76	9.14	4.75	16.85	16.00
16:0	17.08	17.08	9.39	2.72	14.22	13.49
16:1	1.76	1.76	2.25	2.03	2.06	1.83
18:0	0.24	0.24	1.17	0.44	0.61	0.29
18:1	9.01	9.01	20.93	22.86	14.58	12.47
18:2	0.41	0.41	0.00	0.72	0.58	0.49
18:3n-3	0.14	0.14	0.13	0.09	0.05	0.13
20:4n-6	0.08	0.08	0.00	0.09	0.00	0.08
20:5n-3	0.05	0.05	0.07	0.00	0.12	0.04
22:6	45.31	45.31	53.37	64.62	47.38	48.79
Other			3.31	1.42	1.89	0.99

[a]From data in Table 6, reconstituted triacylglycerols (TAG) = (position 1 + position 2 + position 3)/3.
[b]Calc. 1,2(2,3)diacylglycerols (DAG) = (3 × TAG + *sn*-2-MAG)/4.
[c]*sn*-2-Monoacylglycerols (MAG) from pancreatic lipolysis.

preferentially associated with the *sn*-3 position, with smaller amounts in *sn*-1 position. In contrast, the modest amounts of 24:0 were divided nearly equally between the *sn*-1 and *sn*-3 positions, with very little in the *sn*-2 position. Although the B and A oils are more complex in the composition and positional distribution of the fatty acids, it appears that they conform to the distribution of the polyunsaturated and saturated fatty acids established for the simpler oils (E and F, and C and D series).

Significance. It is difficult to compare the results of this work with published literature because natural oils containing over 33% of long-chain polyunsaturated fatty acids have not been previously subjected to stereospecific analyses. Analyses of TAG of human milk have shown that the 20:4n-6 and

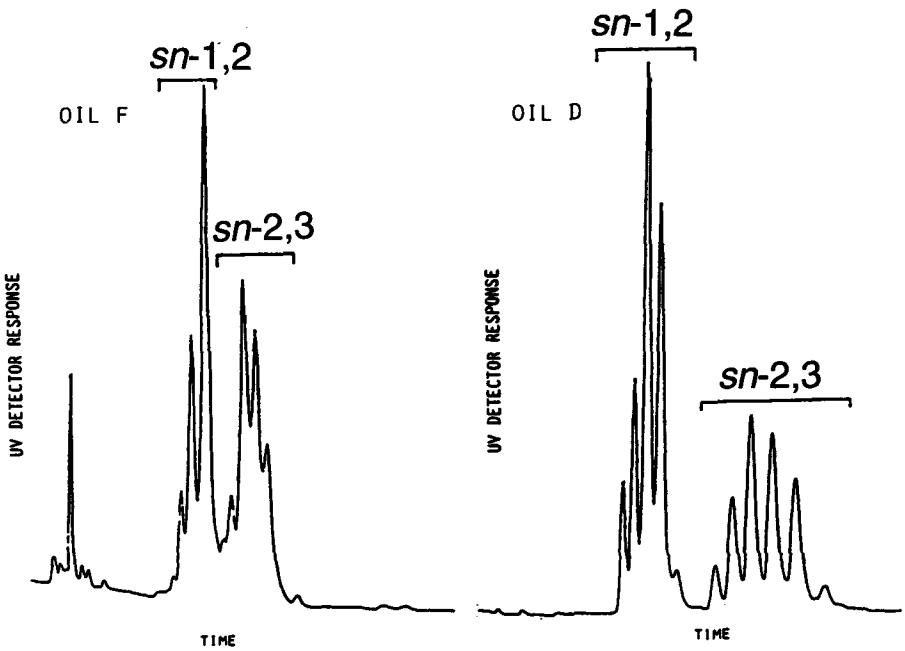


FIG. 4. Chiral-phase HPLC of the diacylglycerol dinitrophenyl urethane derivatives of F and D polyunsaturated triacylglycerols. *sn*-1,2-, *sn*-1,2-diacylglycerols (DAG); *sn*-2,3-, *sn*-2,3-DAG. Chiral-phase HPLC conditions: Hewlett-Packard Model 1090 liquid chromatograph equipped with a chiral column (25 cm × 4.6 mm i.d.) containing R-(+)-1-(1-napthtyl)ethylamine polymeric phase chemically bonded to 300 Å wide-pore spherical silica (YMC-Pack A-KO_3; YMC Inc., Kyoto, Japan). Solvent system: isocratic system of hexane/dichloromethane/ethanol 40:10:1, by vol, at 0.5 mL/min at an oven temperature of 23°C. Ultraviolet detector at 254 nm. See Figure 1 for abbreviations and company source address.

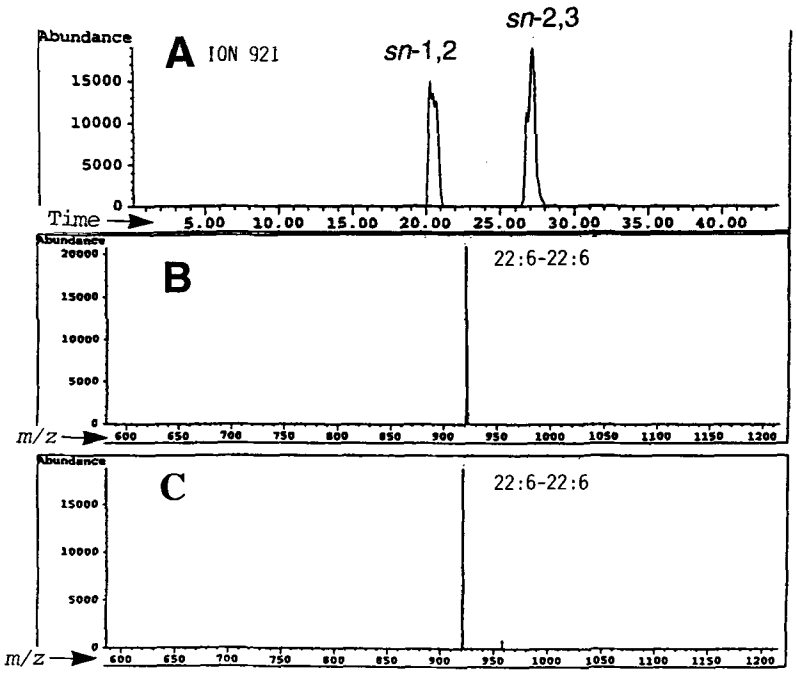


FIG. 5. Chiral-phase liquid chromatography/mass spectrometry (LC/MS) of the dinitrophenylurethane derivatives of oil C polyunsaturated *sn*-1,2- and *sn*-2,3-DAG recovered in the X-1,3-DAG fraction during borate thin-layer chromatography. Chiral-phase HPLC conditions as in Figure 4. LC/MS conditions as given in Experimental Procedures section. A, selected ion [*m/z* 921] profile corresponding to 22:6-22:6 species of DAG; B, total ion spectrum of peak corresponding to *sn*-1,2-enantiomers; C, total ion spectrum of peak corresponding *sn*-2,3-enantiomers. See Figures 1 and 4 for other abbreviations.

22:6n-3 acids are located mainly in the *sn*-2 and *sn*-3 positions (17,18), with the *sn*-1 position being relatively deficient in these acids (17). These findings have led to the observation that the fish oil and mother's milk on digestion would provide polyunsaturated 2-MAG, which would promote the recovery of these acids in the chylomicron TAG (19). On this basis, the oils rich in 20:4 (E and F) and 22:6 (C and D) would also yield high proportions of n-3 and n-6 polyunsaturated 2-MAG. In contrast to mother's milk, the present oils contain also high proportions of polyunsaturated fatty acids in the *sn*-1 position of the glycerol molecule. The metabolic significance of the location of the polyunsaturated fatty acids in the *sn*-1 position of the glycerol molecule is not known. Sample A oil is unusual in the content of comparable proportions of 20:4 in all three positions of the glycerol molecule. It would probably resemble the E and F in its metabolic fate.

These oils enriched in 20:4 and 22:6 acids also possess the unusual feature of containing significant amounts of TAG with two polyunsaturated long-chain fatty acids per molecule. In short-term incubations, pancreatic lipase discriminates against polyunsaturated long-chain fatty acid esters (20,21), but it is not known if this discrimination is more serious when two or three long-chain acids are present in a TAG molecule. During the present stereospecific analyses, there was a clear evidence for discrimination against the polyunsaturated TAG at early times, while at later times 2-MAG of representative composition were obtained.

The positional location and molecular association of long-chain polyunsaturated fatty acids may be pertinent to the resistance of these oils to peroxidation. Recent work has suggested (22) that a polyunsaturated fatty acid in the 2-position is more vulnerable to peroxidation than a similar fatty acid in the primary position. Assuming a fork shape for the TAG molecule, polyunsaturated fatty acids in the primary positions were thought to interact for mutual protection against oxidation. These observations are contradictory to earlier findings, showing that polyunsaturated acids in the primary positions were more vulnerable to peroxidation (23,24). Perhaps the protective interaction between the polyunsaturates in the primary positions is observed only when both are present in the same TAG molecule. The effect of the structural features of these unusual oils upon intestinal digestion and absorption, and upon peroxidation are subject to experimental determination and correlation with the positional distribution of the fatty acids.

ACKNOWLEDGMENTS

These studies were completed with partial support from the Heart and Stroke Foundation of Ontario and the Medical Research Council of Canada.

TABLE 5
Averaged Positional Distribution of Fatty Acids in the Purified Triacylglycerols of E and F Oils (mole %)

Fatty acids	E			F		
	sn-1[a]	*sn*-2	*sn*-3[a]	*sn*-1[a]	*sn*-2	*sn*-3[a]
14:0	0.45	0.23	0.45	0.31	0.30	0.44
15:0	0.11	0.06	0.20	0.16	0.06	0.19
16:0	7.15	1.25	15.62	6.09	1.36	20.47
16:1n-7	–0.09	0.40	–0.07	–0.04	0.30	–0.03
17:0	0.25	0.04	1.08	0.21	0.06	1.35
18:0	5.28	1.05	27.20	4.65	1.12	26.96
18:1n-9	8.60	29.52	5.38	3.66	17.97	2.40
18:1n-7	0.48	0.16	0.26	0.41	0.16	0.31
18:2n-6	1.87	16.70	2.07	0.56	15.11	1.38
18:3n-6	2.15	4.82	1.83	2.02	6.08	1.63
20:0	0.30	0.07	2.81	0.34	0.08	2.93
18:3n-3	0.02	0.60	0.09			
20:1n-9	0.42	0.13	0.71	0.38	0.17	0.94
20:2n-6	0.30	0.71	0.84	0.40	0.78	1.04
20:3n-6	1.28	3.03	2.03	1.43	4.33	2.27
22:0	1.71	0.15	3.83	2.03	0.11	3.74
20:4n-6	66.60	39.14	32.16	72.37	49.95	30.74
20:5n-3	0.26	0.12	0.29	0.37	0.04	0.36
24:0	1.20	0.09	1.81	3.19	0.10	1.15
24:1	0.28	0.00	0.28			
22:6	0.03	0.08	0.03	0.06	0.00	0.06
Other	1.26	1.65	1.03	1.40	1.97	1.65

[a]Average of the two calculations given in text for *sn*-1 and *sn*-3 positions, respectively; composition of *sn*-2 position was not averaged.

TABLE 6
Averaged Positional Distribution of Fatty Acids in the Purified Triacylglycerols of C and D Oils (mole %)

Fatty acids	C			D		
	sn-1[a]	*sn*-2	*sn*-3[a]	*sn*-1[a]	*sn*-2	*sn*-3[a]
10:0	0.27	0.00	0.27	1.12	0.00	1.12
12:0	1.50	0.94	7.82	8.76	0.44	10.22
14:0	24.18	5.44	14.77	30.53	4.75	23.99
15:0	–0.05	0.22	0.00	–0.29	0.18	0.15
16:0	29.68	2.91	13.34	29.81	2.72	18.70
16:1n-7	1.23	2.33	1.20	1.83	2.03	1.42
17:0	–0.01	0.04	0.22	-0.03	0.00	0.06
18:0	–0.01	0.53	4.53	0.84	0.44	–0.57
18:1n-9	3.02	31.54	9.35	3.90	22.86	0.26
18:2n-6	0.36	2.51	1.37	–0.35	0.72	0.86
20:0	0.03	0.00	0.52	–0.18	0.00	0.25
18:3n-3	0.08	0.14	0.16	0.22	0.09	0.11
20:1n-9	–0.04	0.12	0.34	0.09	0.00	0.09
20:3n-6	0.12	0.33	0.48			
22:0	0.20	0.12	0.64	0.03	0.00	0.03
20:4n-6	8.10	4.27	6.97	0.07	0.09	0.07
24:0	0.33	0.07	0.16			
24:1	0.12	0.00	0.12			
22:6	29.84	47.09	34.68	23.58	64.62	42.34
Other	0.89	1.47	3.06	0.78	1.06	0.33

[a]Average of the two calculations given in text for *sn*-1 and *sn*-3 positions, respectively; composition of *sn*-2 position was not averaged.

TABLE 7
Averaged Positional Distribution of Fatty Acids in the Purified Triacylglycerols of B and A Oils (mole %)

Fatty acids	B			A		
	sn-1[a]	*sn*-2	*sn*-3[a]	*sn*-1[a]	*sn*-2	*sn*-3[a]
12:0	0.13	0.13	–0.12			
14:0	3.86	5.17	2.20	0.68	0.16	0.24
15:0	1.71	1.62	0.60	0.74	0.06	–0.27
16:0	35.83	21.31	10.44	23.16	3.15	15.49
16:1n-7	7.33	3.89	4.11	1.68	0.63	–1.68
17:0	4.08	2.30	0.50	0.42	0.09	0.56
18:0	12.18	2.50	2.75	5.72	1.59	11.97
18:1n-9	16.48	6.17	18.47	9.55	20.94	13.19
18:2n-6	1.63	1.33	1.87	12.15	35.67	16.75
18:3n-6	0.02	0.22	0.18	1.96	3.32	4.23
20:0	0.40	0.14	0.79	0.16	0.16	1.64
18:3n-3	0.32	0.35	0.79	2.29	2.28	1.83
20:1n-9	0.80	0.61	1.84	0.10	0.14	1.36
20:2n-6	0.22	0.25	0.43	0.57	0.55	0.20
20:3n-6	0.15	0.09	0.15	2.15	4.79	2.35
22:0	0.26	0.10	0.42	1.93	0.50	3.10
20:4n-6	0.84	2.87	2.79	25.02	22.70	26.54
20:3n-3				–0.45	0.58	1.51
20:5n-3	3.34	6.81	11.69			
24:0				4.83	0.68	3.17
24:1n-9	0.40	0.11	0.76			
22:6	9.07	43.57	39.26	–0.02	0.09	–0.02
Other	1.10	0.20	0.03	2.57	1.92	0.13

[a]Average of the two calculations given in text for *sn*-1 and *sn*-3 positions, respectively; composition of *sn*-2 position was not averaged.

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Quantitative High-Performance Liquid Chromatography Analysis of Plant Phospholipids and Glycolipids Using Light-Scattering Detection

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ABSTRACT: Application of the evaporative light-scattering principle to quantitative high-performance liquid chromatography (HPLC) analyses of plant membrane lipids has received little study. Light-scattering detection response curves were generated for nine classes of plant membrane phospholipid and glycolipids. Quantitative results obtained by HPLC/light-scattering detection and conventional lipid analytical methods (thin-layer chromatography and lipid-P assay) were in close agreement, confirming the reliability of HPLC/evaporative light-scattering detection (ELSD) analyses. Only three of the nine plant lipid classes gave linear detector response functions above 10 μg injected lipid mass. This finding contradicts earlier precepts involving light-scattering detection of lipids. At a given mass, appreciable variation in ELSD signal intensity and detection limit was found to exist among the various plant membrane lipid classes. The variation in detector response among plant lipid classes is an important consideration in achieving accurate quantitative results in plant lipid analyses.
Lipids 31, 217–221 (1996).

The availability of an efficient method to quantify plant phospholipid and glycolipid classes would aid in our understanding of the vital functions that these membrane structural components fulfill in plant tissues. Traditionally, thin-layer chromatography (TLC) has been used to isolate and quantify complex lipids, but quantification may be time-consuming, tedious, and labor-intensive (1,2).

High-performance liquid chromatography (HPLC) has become an important tool for the separation and analysis of phospholipids and glycolipids. Since total plant lipid extracts are diverse, gradient elution procedures are necessary to achieve effective separations (3), and this may present detection difficulties in quantitative analyses. These problems have been discussed in detail, particularly regarding ultraviolet, refractive index, and fluorescence detection of lipids (4,5). The recent introduction of evaporative light-scattering (ELS) detectors, which are largely unaffected by changes in mobile phase composition, has surmounted these difficulties and offers new opportunities for quantitative lipid class analyses (6).

Several applications of HPLC/ELS detection in quantitative analyses of neutral and polar lipid classes from animal tissues are available (5,7,8). More recent accounts of HPLC separation and ELS detection have included the analysis of phospholipids from seed oils (6,9) and of both phospholipids and glycolipids from wheat flour (10). Such reports reveal major differences in lipid class profiles between these sources and higher plant tissues. Quantitative data for phospholipid and glycolipid classes in plant tissues obtained by using ELS detection are unavailable. Moreau (11) demonstrated that this detection technique may be useful in neutral lipid, glycolipid, and phospholipid separation of plant tissues, but this report did not provide quantitative analyses.

It has been demonstrated that ELS detector sensitivity may vary among characteristic animal lipid classes (5). This finding could have an important bearing on quantification of plant lipid classes, particularly those present in low concentrations in tissue. The objectives of this study were to evaluate possible variation among plant phospholipid and glycolipid classes in ELS detector response, and to compare quantitative analyses of plant membrane lipid classes between HPLC/ELSD and conventional lipid analytical procedures.

MATERIALS AND METHODS

Plant material and sample preparation. Carrots (*Daucus carota* L. "Apache") were purchased from a local produce distributor and mature apple fruit (*Malus domestica* Borkh. "Red Delicious" and "Golden Delicious") were obtained from a nearby orchard.

Peeled and shredded carrots, and the outer 2–3 mm of cortical tissue of apple fruit (obtained after peeling the fruit) were frozen in liquid N_2 and lyophilized. Total lipid extracts were obtained from dried, powdered subsamples (200 mg for

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Abbreviations: ASG, acylated sterol glycoside; DGDG, digalactosyldiacylglycerol; ELSD, evaporative light-scattering detection; FID, flame-ionization detection; HPLC, high-performance liquid chromatography; LPC, lysophosphatidylcholine; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SG, sterol glycoside; TLC, thin-layer chromatography.

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carrot, 500 mg for apple) which were homogenized in 10 mL chloroform/meth-anol (2:1, vol, vol), then washed using the methods of Folch *et al.* (12). The total lipid extract was separated into glycolipid and phospholipid fractions using solid-phase extraction, and these fractions were prepared for HPLC analyses as described previously (13,14).

HPLC system. Component classes in glycolipid and phospholipid fractions were separated by normal-phase HPLC using a 10 cm × 3 mm, 5-μm ChromSep LiChrosorb Si 60 silica cartridge (Chrompak, Raritan, NJ). Samples (100 μL) were introduced using a WISP 712 programmable injector (Waters, Milford, MA). The injector also served to initialize a Waters 600E solvent delivery system and personal computer for data acquisition at the beginning of each analysis. Maxima 820 software (Dynamics Solutions, Millipore Corp., Ventura, CA) was used for peak integration, generation of calibration curves and regression coefficients, and the determination of plant lipid class concentrations.

The mobile phases for glycolipid and phospholipid elutions were modified from the ternary gradient systems developed by Moreau *et al.* (15) and Letter (16) using 2-propanol, hexane, and water, with the elution schemes described previously (13,14). All solvents were continuously sparged with He (20 mL/min), and the solvent mixture was maintained at a flow rate of 0.5 mL/min.

Lipid classes were detected using a Varex IIA ELS detector (Varex Corp., Burtonsville, MD). The N_2 flow rate was maintained at 2.5 L/min, and the drift tube temperature was held at 90°C throughout all analyses. Calibration curves were generated by injecting a concentration series of authentic lipid class standards. Calibration data were log-transformed, and comparisons of the slopes and intercepts from the various lipid classes were made using the method of Snedecor and Cochran (17) on MSTAT-C, version 2.11 (Michigan State University, East Lansing, MI).

Solvents and lipid calibration standards. Phospholipid standards [phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC), lysophosphatidylcholine (LPC), and phosphatidic acid (PA)] were obtained from Sigma Chemical Co. (St. Louis, MO) and were derived from soybean, except for PA which was derived from egg yolk lecithin. Preliminary analyses on HPLC confirmed that the commercial source of PA coeluted with PA isolated from plant tissue by TLC.

Glycolipid standards [monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sterol glycoside (SG), and acylated sterol glycoside (ASG)] were purchased from Matreya (Pleasant Gap, PA) and were of plant origin (plant species unknown). MGDG and DGDG were completely hydrogenated (saturated acyl groups). Preliminary comparisons with highly unsaturated MGDG and DGDG (isolated from plant tissue by TLC) showed that HPLC separation and quantification of MGDG and DGDG were unaffected by fatty acid unsaturation.

HPLC-grade solvents (2-propanol and hexane) were from Mallinckrodt Specialty Chemicals, Inc. (Paris, KY). Water was purified using a Nanopure II system (Barnstead, Boston, MA), then passed through a 0.22-μm Nitroplus cellulose filter (Micron Separations, Inc., Westboro, MA) before use.

TLC and lipid-P analyses. Quantitative tissue analyses of HPLC and one-dimensional TLC analyses were compared for ASG, SG, and MGDG. Aliquots of glycolipid fractions were loaded onto 10 × 20 cm TLC plates coated with a 0.25-mm thickness of silica gel 60 (EM Science, Cherry Hill, NJ). Glycolipids were separated using a solvent mixture of chloroform/ethyl acetate/methanol/acetic acid/water (120:20:40:20:4, by vol). All TLC solvents were HPLC-grade and were obtained from Burdick and Jackson (Muskegon, MI). Spots were identified, eluted from the gel, and the lipids quantified as described by Whitaker (18).

Total lipid-P was determined on duplicate, 20-μL aliquots of the plant phospholipid fractions by the method of Ames (19). The total phospholipid mass in each sample was then calculated using an average molecular weight of 750.

Results obtained from TLC (glycolipid fractions only) and total lipid-P determinations were compared with quantitative HPLC results using student's *t*-tests and linear regression, respectively.

RESULTS AND DISCUSSION

Relationship between lipid class and ELS detector response. Second-order polynomial or linear regression calibration curves provided regression coefficients (r^2) of 0.98 or greater for all lipid classes (data not shown), but detector response varied between classes (Figs. 1 and 2). At masses greater than approximately 10 μg, linear response functions were obtained only for PI, PC, and DGDG. The remaining lipid classes (PE, LPC, PA, MGDG, SG, and ASG) produced exponential functions at masses up to 80 μg. Although we could find no prior documentation of calibration curves for each of these lipid classes in a single report, this finding contradicts the generalization that ELS detectors provide linear responses for lipids at masses of 10 μg and above (4,10,16).

Log-transformed data of Figures 1 and 2 provided linear functions for each lipid class, but the slopes were not parallel (Table 1). The lipid classes yielding exponential curves above 10 μg had greater slopes on log/log plots than did lipid classes giving linear curves above 10 μg. The greater slopes for PA and ASG were the result of relatively low detector sensitivity for these classes at low injected masses as compared to the other lipid classes in their respective groups (see insets in Figs. 1 and 2). This is further indicated by the low y-intercept values for PA and ASG. Peak areas of PA and ASG never reached the levels of their counterpart lipid classes even with 80-μg injections, indicating relatively low detector sensitivity for these lipid classes.

Variation in ELSD response among lipid classes at equivalent masses may partly result from differences in lipid-specific particle sizes which result in different amounts of light scattering (16). Such variation should be expected when considering the large molecular size variation among the lipid

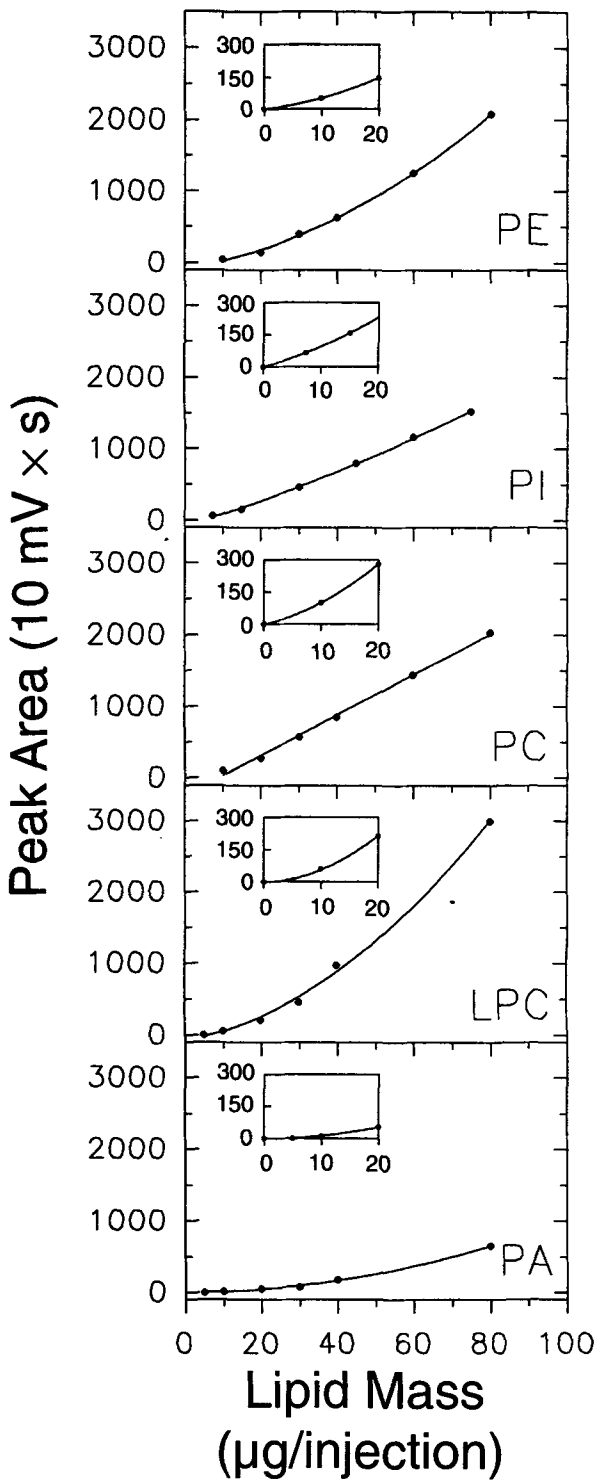


FIG. 1. Phospholipid class calibration curves of mass injected vs. detector response. Each point represents the mean of duplicate injections. Insets are expanded portions of the curves below 20 µg. PE, phosphatidylethanolamine; PI, phosphatidylinositol; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PA, phosphatidic acid.

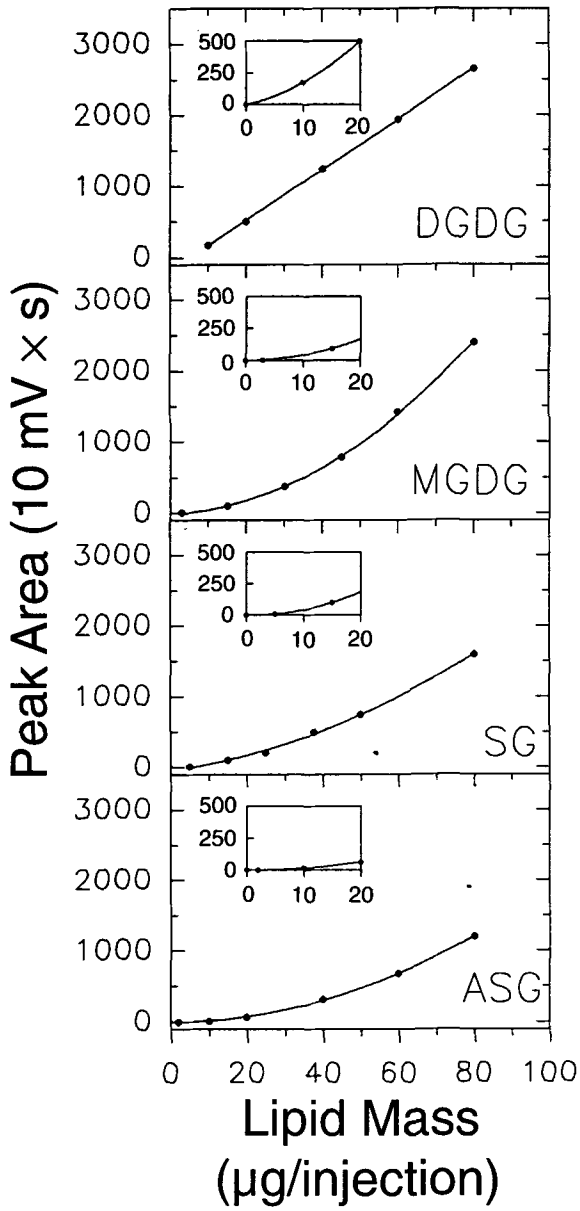


FIG. 2. Glycolipid class calibration curves of mass injected vs. detector response. Each point represents the mean of duplicate injections. Insets are expanded portions of the curves below 20 µg. DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; SG, sterol glycoside; ASG, acylated sterol glycoside.

classes analyzed in our study (up to 45% differences in formula weights).

Detection limits for PE, PI, PC, LPC, DGDG, MGDG, and SG ranged between 250 and 500 ng. However, the detector showed reduced sensitivity and lower peak height/background ratios for PA and ASG, and we were unable to obtain reliable detection below 1 µg for these two classes. Lower limits of lipid detection with ELSD have been documented (5,16), but these reports included analyses of neutral lipid classes. Neutral lipids elicit greater ELSD responses than polar lipids (8); thus these studies did not fully assess the potential of ELS detection for the variety of plant lipid classes used in our study.

Lipid quantification by HPLC/ELSD and conventional procedures. Quantitative HPLC results for glycolipids were

TABLE 1
Linear Regression Coefficients from Log-Transformed Data in Figures 1 and 2[a]

Lipid class	Y-intercept	Slope	r
Phospholipids			
PI	0.58 a	1.41 b	0.999
PC	0.54 a	1.46 b	0.999
PE	−0.12 a	1.81 a	0.998
LPC	−0.58 a	2.19 a	0.991
PA	−0.75 b	1.85 a	0.996
Glycolipids			
DGDG	0.95 a	1.32 c	0.998
MGDG	−0.28 b	1.93 b	0.999
SG	−0.32 c	1.88 b	0.997
ASG	−1.17 d	2.26 a	0.999

[a]Values within columns and lipid class groups with the same letter are not significantly different by F-test ($P \leq 0.05$). PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; LPC, lysophosphatidylcholine; PA, phosphatidic acid; DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; SG, sterol glycoside; ASG, acylated sterol glycoside.

TABLE 2
Glycolipid Class Concentrations of "Red Delicious" Apple Fruit Determined After One-Dimensional TLC and After HPLC[a]

Glycolipid class	Glycolipid concentration (mg/100 g dry tissue weight) HPLC	TLC
ASG	6.7 ± 0.5 (7.5)	7.7 ± 1.6 (20.7)
SG	29.7 ± 1.5 (5.1)	26.4 ± 3.3 (12.5)
MGDG	20.2 ± 1.3 (6.4)	19.0 ± 3.6 (18.9)[b]

[a]Values are the mean ± standard deviation of three determinations on separate fruit, each representing a paired analysis of the same tissue sample. Means between columns are not significantly different by *t*-test at the 5% level. Numbers in parentheses refer to coefficient of variation (%). HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography. See Table 1 for other abbreviations.
[b]Mass determined by multiplying molar values obtained following spectrophotometric assay (Ref. 18) by a molecular weight of 772.

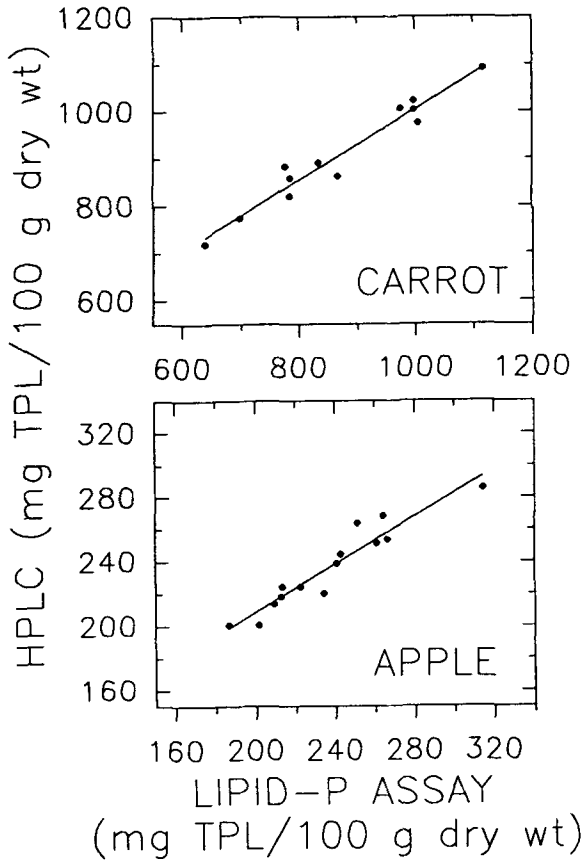


FIG. 3. Total phospholipid mass (TPL) of "Apache" carrot and "Golden Delicious" apple tissues determined by lipid-P assay and by summation of individual phospholipid masses determined by high-performance liquid chromatography (HPLC) peak integration. Regression equations as follows: $y = 256 + 0.75x$, $r = 0.97$, $N = 12$ (carrot); $y = 61 + 0.74x$, $r = 0.95$, $N = 14$ (apple). Each point represents a paired analysis of the same tissue sample.

in close agreement with one-dimensional TLC analyses (Table 2). Method averages did not differ significantly ($P \leq 0.05$), but variability was 2.5 to 3 times greater for the TLC averages. The increased variability with TLC probably resulted from the greater number of sources of systematic error, such as in the quantitative scraping and eluting of silica gel and in the subsequent spectrophotometric assay.

Total phospholipid values obtained by conventional lipid-P analyses and by summation of individual phospholipid masses by HPLC peak integration were closely correlated (Fig. 3). The total mass of phospholipid determined by lipid-P analysis (as compared with HPLC analysis) averaged 95.6 ± 5.3% for carrot tissue and 100.1 ± 4.7 for apple tissue. The wide ranges of total phospholipid concentrations in both tissues were the result of endogenous phospholipid increases which occurred during previous experiments (13,14).

Quantitative HPLC data were reproducible, with coefficients of variation ranging from 1 to 5% during automated analyses lasting up to 48 h. Phillips *et al.* (20) reported a close correlation between HPLC/FID and TLC quantitative analyses of animal lipid classes. However, FID systems are more expensive and require more maintenance than ELSD (11). In addition, a new version of the Varex ELS detector (Model III) may provide greater detection sensitivity over FID detectors as well as the version used in our study (21; R. Moreau, personal communication).

In conclusion, results of this study confirm the usefulness of ELS detection in the quantitative analyses of plant membrane phospholipid and glycolipid classes. For most of the plant lipid classes evaluated, detection is a nonlinear function above approximately 10 to 20 μg of lipid mass. Also, ELS sensitivity and detection limits appear to vary ELSD among plant lipid classes. These findings, which have been largely overlooked to date, are important considerations in achieving accurate quantitative results in plant lipid analyses.

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Phosphatidylglycerol Molecular Species of Photosynthetic Membranes Analyzed by High-Performance Liquid Chromatography: Theoretical Considerations

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ABSTRACT: A reversed-phase high-performance liquid chromatography technique was developed to separate, identify, and quantify individual phosphatidylglycerol (PG) molecular species in thylakoid membranes isolated from higher plant leaves. PG was first separated by thin-layer chromatography; then the dinitrobenzoyl derivatives of diacylglycerols produced after phospholipase C hydrolysis of PG were separated by a C_{18} reversed-phase column and detected at 254 nm. A linear response of the detector was observed in the range of 0.025 to 12 nmol of PG molecular species. It was established that there was an excellent correlation (r = 0.996) between the carbon and double-bond number in the aliphatic residues and the relative retention time of dinitrobenzoyl derivatives. A new equivalent carbon number value (ECN*) which takes into consideration the number of *cis*-(n_c) and *trans*-(n_t) double bonds per molecular species was defined as ECN* = CN − $2n_c$ − n_t, where CN is the number of carbon atoms in the aliphatic residues. The logarithm of the retention time increased linearily as a function of ECN* value. However, in this type of correlation, it may happen that two molecular species of PG having distinct relative retention times had the same ECN* value. In this case, the two molecular species can be identified by the linear correlation (r = 1) existing between the reciprocal of the relative retention time and the number of double bonds ($0 \leq n \leq 3$) in the separate 18:n/Δ3-*trans*-hexadecenoic acid [16:1(3*t*)] and 18:n/16:0 molecular species series. The advantages of this method are good separation, short elution time, quantitative precision, and predictable retention times of PG molecular species from chloroplast membranes. The method has been used routinely to identify the ten PG molecular species of thylakoid membranes in squash, potato, lettuce, and spinach leaf: 18:3/16:1(3*t*), 18:3/16:0, 18:2/16:1(3*t*), 18:2/16:0, 18:1/16:1(3*t*), 18:1/16:0, 18:0/16:1(3*t*), 18:0/16:0, 16:0/16:1(3*t*), and 16:0/16:0.

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Phosphatidylglycerol (PG) represents about 10 mol% of the total acyl lipids in thylakoid membranes of higher plants (1). In spite of its small proportion, this lipid is characterized by several unique and important features. First, PG is thought to be the only anionic phospholipid in higher plant thylakoid membranes (2). It is asymmetrically distributed across the membrane with a significant enrichment (69 mol%) in the outer monolayer (3). It also contains a special fatty acid, the Δ^3-*trans*-hexadecenoic acid [16:1(3*t*)], which is always linked to the glycerol molecule at the *sn*-2 position (4,5): There is evidence that 16:1(3*t*)-containing PG is involved in the molecular organization of the LHCII receptor complex (6). We have recently found that the phospholipid population which is located in the inner monolayer sustains most of the uncoupled noncyclic electron flow activity (3,7). Finally, because of its high-melting point fatty acids [16:0, 18:0, and 16:1(3*t*)] in plants sensitive to chilling, PG can form a gel phase in the membrane at cold temperatures and, consequently, induce chilling damage (8,9).

Thus far, most of the information about PG concerns its fatty acid composition. However, molecular species may be even more important than total fatty acid composition in the structure/function relationship of this lipid. Subtle differences in molecular species composition significantly may affect the physical properties and physiological activities of a membrane (10).

In order to separate, identify, and quantify the molecular species of PG, several methods have been proposed in the literature, e.g., thin-layer chromatography (TLC) on $AgNO_3$ in silica gel (5,11), capillary column gas chromatography using volatile derivatives and coupled gas chromatography/mass spectrometry (12). However, the argentation method cannot be used to resolve certain PG molecular species such as PG 16:0/16:0, 16:0/16:1(3*t*), 18:0/16:0, and 18:0/16:1(3t) (5). Kito *et al.* (13) have developed a sensitive method for the quantitative analysis of phospholipid molecular species by high-performance liquid chromatography (HPLC). Diacylglycerols (DAG) were prepared from phospholipids by phospholipase C (PLC) treatment and converted to the corresponding dinitrobenzoyl (DNB) derivatives which were detected at 254 nm. This method has been successfully used to analyze the molecular species of phosphatidylcholine and phosphatidylethanolamine extracted from animal cells (14).

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Abbreviations: DAG, diacylglycerol; DNB, dinitrobenzoyl; ECN, equivalent carbon number; HPLC, high-performance liquid chromatography; PG, phosphatidylglycerol; PLC, phospholipase C; RRT, relative retention time; TLC, thin-layer chromatography; 16:1(3*t*), Δ^3-*trans*-hexadecenoic acid.

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Using the method described by Kito's group (13,14), we report here on the quantitative separation of PG molecular species in thylakoid membranes isolated from plant leaves. Based on the relationship between the number of carbon atoms as well as of double bonds and the relative retention time (RRT) of PG DNB derivatives, the corresponding molecular species of PG can be identified.

EXPERIMENTAL PROCEDURES

Chemicals. PLC, EC. 3.1.4.3 (grade 1, from *Bacillus cereus*) was from Boehringer Mannheim (Rotkreuz, Switzerland). Pyridine and 3,5-DNB chloride were provided by Fluka Chemie AG (Buchs, Switzerland), and diethyl ether extra pure, acetonitrile, and 2-propanol (gradient grade) were from Merck (Darmstadt, Germany). All other solvents were of reagent grade.

Plant material. Squash (*Cucurbita pepo* L. cv Quintal), potato (*Solanum tuberosum* L. cv Granola), and spinach (*Spinacia oleracea* L. cv Nobel) plants were grown in a growth chamber under the following conditions: 12 h of light (350 μmol • s^{-1} • m^{-2})/22°C/50% relative humidity and 12 h darkness/18°C/60% relative humidity. For squash plants, the two first leaves were harvested when the third leaf appeared (about two weeks after germination). For potato and spinach plants, mature leaves were harvested after about one month of growth. Lettuce (*Latuca sativa* L. cv Laitue Romaine de Morges) leaves were from the local market.

Preparation of thylakoid membranes. Deribbed leaves (about 30 g) were ground in a Waring Blender for 5 s in 160 mL of a grinding medium containing 330 mM sorbitol/30 mM 4-morpholinopropanesulfonic acid–KOH (pH 7.8)/2 mM. The mixture was filtered through six cheesecloth layers; then the filtrate was spun down at 1910 × *g* for 1 min. The supernatant was discarded by aspiration while the intact chloroplast pellet was resuspended in a lysis medium containing 10 mM *N*-Tris[hydroxymethyl]methylglycine–KOH (pH 7.8) first in 4 mL with a smooth brush, then in 30 mL. After 1 min, isomolarity was restored by adding 4 mL of 3 M sorbitol. The mixture was spun down at 4300 × *g* for 2 min. After discarding the supernatant by aspiration, thylakoids were resuspended in a washing medium containing 300 mM sorbitol/10 mM *N*-Tris[hydroxymethyl]methylglycine–KOH (pH 7.8) then sedimented for 2 min at 4300 × *g*. The thylakoid pellet was resuspended in the same washing medium and adjusted to 1 to 2 mg chlorophyll/mL. The chlorophyll concentration was determined according to Bruinsma (15).

Lipid extraction. To 1 mL of thylakoid suspension (1 to 2 mg chlorophyll/mL), 4 mL of chloroform/methanol (1:1, vol/vol) and 0.8 mL of 100 mM KCl were added. The mixture was centrifuged (1300 × *g* for 5 min). The lower phase was collected and dried under N_2. The pellet was resuspended in chloroform/methanol (8:2, vol/vol).

PG separation. The acyl lipids contained in the chloroform/methanol extract were separated by two-dimensional TLC on silica gel 60 plates (pre-coated silica gel plates, Merck 5626). The total lipid extract corresponding to 0.15 mg chlorophyll was spotted on the plate using an automatic sample applicator (Linomat IV; Camag, Switzerland). TLC plates were developed in acetone/toluene/water (91:30:8,by vol). After drying in an N_2 atmosphere, TLC were developed in the second dimension with a mobile phase consisting of chloroform/methanol/25% NH_3/water (65:35:2:3,by vol). Lipids were revealed with 0.01% primuline in acetone/H_2O (60:40, vol/vol) and the band corresponding to PG was scrapped and introduced into a glass tube. After the addition of 30 μg of distearoyl phosphatidylcholine as internal standard, the mixture was washed twice with chloroform/methanol (1:1, vol/vol) to eliminate silica gel then dried under N_2.

PLC treatment and preparation of DNB derivatives. The removal of the polar head groups of PG was achieved by treatment with the PLC and the preparation of the 3,5-DNB derivatives of PG was carried out according to the method of Kito *et al.* (13), however, with some modifications. The purified PG was resuspended by sonication in 1.25 mL of 10 mM Tris–HCl (pH 7.5) containing 30 mM H_3BO_3, 1 mM $ZnSO_4$, and 5 mM $CaCl_2$. After the addition of ten units of PLC (5 μL) and 2 mL of water-saturated diethyl ether, the tubes were shaken at 30°C for 60 min. The diethyl ether layer containing DAG was collected, whereas the remaining aqueous phase was washed twice with 2 mL of water-saturated diethyl ether. The diethyl ether fractions were then dried under N_2.

HPLC analysis of 3,5-DNB derivatives. HPLC was performed on a Kontron HPLC-system 400 with detection at 254 nm. The column used was the KS250/6/4/nucleosil 100-5C_{18} mounted with a guard column KS 11/6/4/nucleosil 120-7C_{18} from Macherey-Nagel (Düren, Germany). Elution was achieved isocratically with acetonitrile/2-propanol (80:20, vol/vol).

Fatty acid analysis. Each HPLC peak corresponding to 3,5-DNB PG derivative molecular species was collected and dried under N_2 flow. The fatty acid constituents of each PG derivative were transesterified with 5% H_2SO_4 in methanol at 85°C for 1 h under N_2 (1). The extraction of the methyl esters was achieved with 1.5 mL pentane after addition of 1 mL water. The extract was evaporated to dryness with N_2, taken up in 150 μL hexane and injected (5 μL) automatically into the gas chromatograph Hewlett-Packard 5890 (Palo Alto, CA) mounted with a capillary column Hewlett-Packard free fatty acid phase (30 m; i.d. 0.53 mm). The column was isothermically run at 190°C with helium as carrier (14.8 mL/min) while the detector (flame-ionization detector) was held at 230°C. Retention times of fatty acid methyl esters were compared with those of standard methyl esters of 18:0, 18:1, 18:2, 18:3, 16:0, and 16:1(3*t*), allowing the identification of the fatty acids contained in each HPLC peak. The quantification of fatty acids was carried out by adding a known amount (30 μg) of arachidic acid (20:0) in each sample, just before the transesterification step.

RESULTS AND DISCUSSION

Molecular species of thylakoid membrane PG in four plant species. Figure 1 shows the HPLC profile of 3,5-DNB deriv-

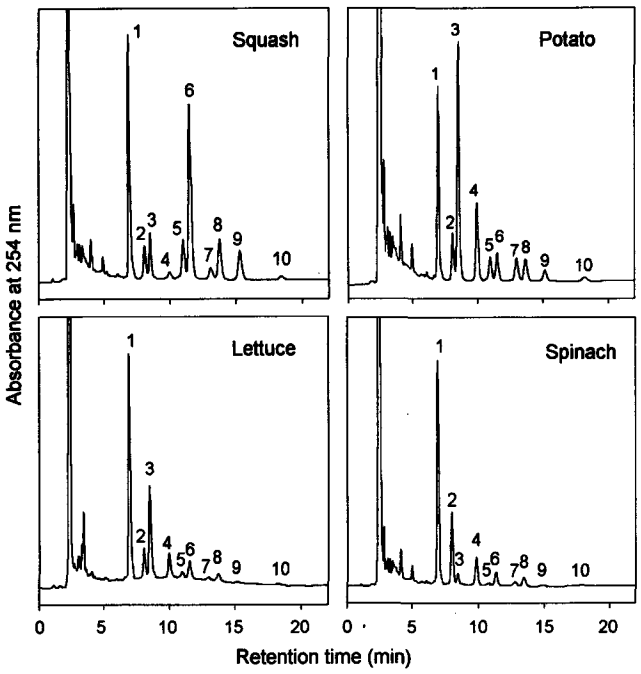


FIG. 1. High-performance liquid chromatography profile of 3,5-dinitrobenzoyl derivatives of phosphatidylglycerol isolated from thylakoid membranes of squash, potato, lettuce, and spinach. Peak numbers correspond to those in Tables 1 and 2. The retention time of the internal standard (3,5-dinitrobenzoyl derivative of 18:0/18:0 phosphatidylcholine) was 25.02 min (data not shown).

atives of DG isolated from thylakoid membranes of four plant species. All thylakoid membranes were characterized by ten HPLC peaks numbered from 1 to 10. It is noteworthy that, under our experimental conditions, all compounds were eluted from the HPLC column during 20 min only. The peaks appearing during the first 7 min were not due to lipid derivatives since an HPLC profile of a control sample (e.g., without added PG DNB derivatives) gave exactly the same profile as those illustrated in Figure 1 (results not shown). In addition, no methyl esters of fatty acids were detected by gas chromatography in the first fractions of the HPLC eluate. As an example, the composition in fatty acids of each HPLC peak from squash thylakoid PG (Fig. 1) is shown in Table 1. Each peak included two main fatty acids, except peak 8, which contained essentially 16:0. Within experimental errors, the amount of these two fatty acids was the same. However, each peak included small amounts of fatty acid contaminants due to manual collection of the eluted sample. In our hands, contaminants never exceeded 0.8 mol% of total PG (e.g., the contamination by 18:1 in peak 6). This indicates that each peak corresponded to one DNB–DAG molecular species. Table 1 also shows that each HPLC peak always contained either 16:0 or 16:1(3*t*) as one of the main fatty acids, the other one being a fatty acid in the C_{18} series, in agreement with the finding of Roughan (5). However, there are two exceptions to this general rule, i.e., peak 6 containing both 16:0 and 16:1(3*t*) and peak 8 containing essentially 16:0 (in double amount). Several authors have reported that in leaf PG of higher plants, the *sn*-1 position is occupied either by a fatty acid of the C_{18} series or by 16:0, whereas the *sn*-2 position always contains a fatty acid of the C_{16} series. This last finding has been verified by hydrolyzing the ester bond of PG at the position *sn*-2 with snake venom phospholipase A_2 according to Chapman and Barber (16). Moreover, when present, 16:1(3*t*) is always located at the *sn*-2 position (4,5,8,17). On the basis of these findings, it was possible to assign a PG molecular species to each of the HPLC peak, as illustrated in Table 1. It can be seen that about 50 mol% of PG in squash (a plant sensitive to chilling) thylakoid membranes contained highly saturated molecular species [e.g., 16:0/16:1(3*t*), 16:0/16:0, 18:0/16:1(3*t*), 18:0/16:0]. In contrast, the PG from potato, and even more from lettuce and spinach (chilling-resistant plants) thylakoid membranes, was characterized by much more unsaturated molecular species [e.g., 18:3/16:1(3*t*), 18:2/16:1(3*t*); see Fig. 1]. These results are qualitatively in agreement with earlier observations (12,18). However, Sekiya *et al.* (19) have reported that in leaves of various *Citrus* species, there are also additional mol-

TABLE 1
Determination by Gas Chromatography of the Fatty Acid Composition and Content of Each High-Performance Liquid Chromatography Peak of Phosphatidylglycerol (PG) Isolated from Squash Thylakoid Membranes

Peak[a]	Fatty acid composition (mol%)						Molecular species	
number	16:0	16:1(3*t*)	18:0	18:1	18:2	18:3	*sn*-1/*sn*-2[b]	Mol% of total PG
1	4.1	47.3[c]		1.3	1.0	46.4	18:3/16:1(3*t*)	28.9
2	48.3	1.4		1.3	1.8	47.3	18:3/16:0	5.5
3	5.2	45.8		1.1	45.3	2.6	18:2/16:1(3*t*)	6.5
4	46.8	3.1		12.7	33.3	4.0	18:2/16:0	1.3
5	6.9	45.9		47.2			18:1/16:1(3*t*)	7.7
6	47.4	48.2	1.7	2.6			16:0/16:1(3*t*)	31.0
7	47.1	5.1		47.8			18:1/16:0	2.5
8	94.7	1.2	3.1	1.1			16:0/16:0	8.6
9	1.8	48.9	49.3				18:0/16:1(3*t*)	7.1
10	48.0	1.8	48.5	1.7			18:0/16:0	1.0

[a]Peak numbers correspond to those indicated in Figure 1.
[b]The *sn* position of fatty acids was not determined but assumed from other reports (see text); 16:1(3*t*), Δ^3-*trans*-hexadecenoic acid.
[c]The underlined numbers correspond to the main fatty acids in each peak.

ecular species, such as 18:3/18:3 and 18:2/18:3. These species are likely to originate from other leaf membranes.

Determination of the DNB–DAG molecular species by their HPLC retention time. Figure 2 shows the relationship between the logarithm of the retention time of 3,5-DNB derivatives of PG isolated from squash thylakoid membranes and the equivalent carbon number (ECN) value of each PG molecular species. The ECN value, which was thoroughly discussed by Christie (20), is defined as the actual number of carbon atoms in the aliphatic residues (CN) less twice the number of double bonds (n) per molecule (the carbons of the glycerol moiety are not counted for this purpose), i.e., ECN = CN – 2n. Though the ECN value was applied to triacylglycerol molecular species (for a review, see Christie, Ref. 20), we found that this relationship also applied to 3,5-DNB derivatives of phospholipids, provided that the influence of *trans*-double bonds, which are frequent in PG molecular species, is considered in a special manner. Based on the results shown in Table 2 and Figure 1, we propose a new formula expressing the actual number of carbon atoms: ECN* = CN – $2n_c - n_t$, where n_c and n_t correspond to the number of *cis*- and *trans*-double bonds per molecular species, respectively. For example, in the 18:3/16:1(3*t*) PG molecular species, ECN* = 34-6-1 = 27. Table 2 also shows that the retention time increased exponentially as a function of the ECN* values. Consequently, the logarithm of the retention time increased (apparently) linearily as a function of ECN* values as illustrated in Figure 2A (y = 0.059 × – 0.748; r = 0.996). However, two molecular species of PG [e.g., 18:1/16:1(3*t*) and 16:0/16:1(3*t*)] presented the same ECN* value (ECN* = 31) but were characterized by two different retention times. Similarly, two other species (18:1/16:0 and 16:0/16:0) also differing by their retention time, had the same ECN* value (ECN* = 32). This similarity can be explained by the fact that the retention time value is a precise experimental data while the ECN* value is an estimation of the ECN for which it was assumed that one *cis*- and one *trans*-double bond corresponded to two and one carbon, respectively. Thus, the two pairs of molecular species which have distinct retention time presented the same ECN* value.

As can be seen in Table 1, the ten PG molecular species, except 16:0/16:0 and 16:0/16:1(3*t*), can fit into the two series:

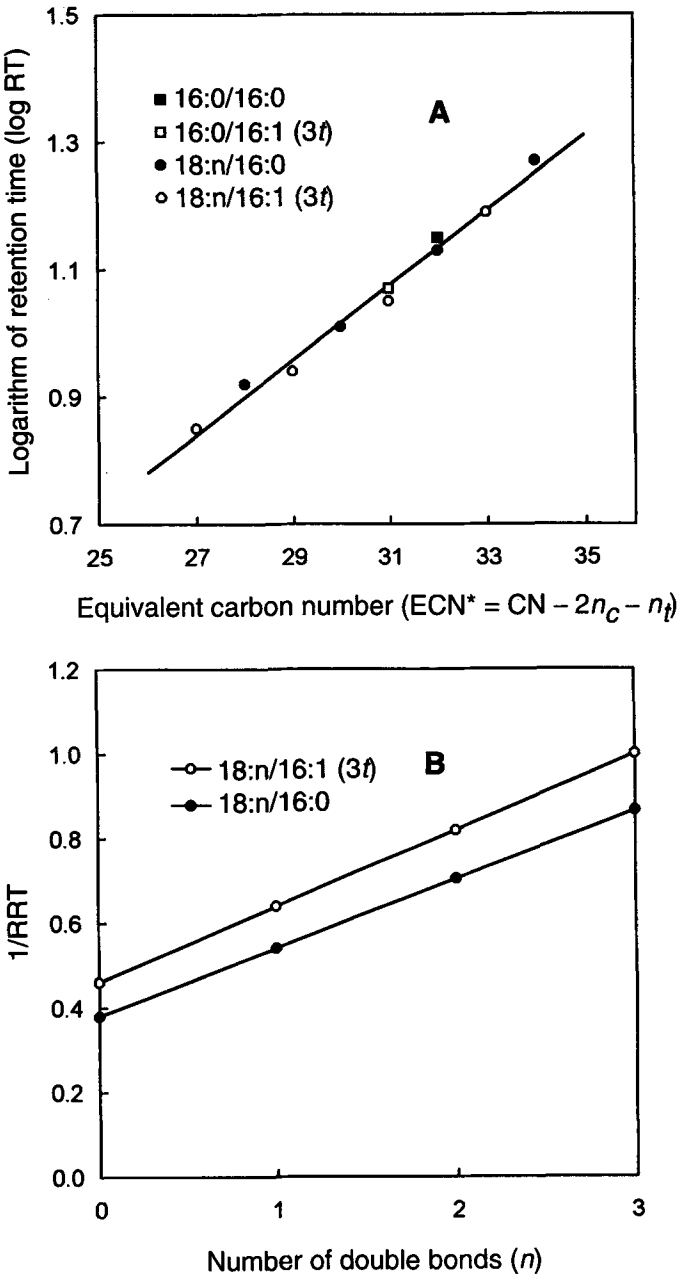


FIG. 2. A: Correlation between the logarithm of the retention time expressed in min (log RT) and the corrected equivalent carbon number (ECN*) in phosphatidylglycerol molecular species of squash thylakoid membranes. ●, 18:n/16:0; ○, 18:n/Δ^3-*trans*-hexadecenoic acid [16:1(3*t*)], ■, 16:0/16:0; □, 16:0/16:1(3*t*). B: Correlation between the reciprocal of the relative retention time (1/RRT) and the number of double bonds (n) in 18:n/16:0 (●) and 18:n/16:1(3*t*) (○) molecular species of phosphatidylglycerol.

TABLE 2
Relationship Between the ECN of PG Molecular Species and the Retention Time of 3,5-Dinitrobenzoyl Derivatives of PG Isolated from Squash Thylakoid Membranes

Peak number[a]	Molecular species	ECN*[b]	Retention time (min)	RRT[c]	1/RRT
1	18:3/16:1(3*t*)	27	7.03	1.00	1.000
2	18:3/16:0	28	8.10	1.15	0.868
3	18:2/16:1(3*t*)	29	8.60	1.22	0.817
4	18:2/16:0	30	9.94	1.41	0.707
5	18:1/16:1(3*t*)	31	11.09	1.58	0.634
6	16:0/16:1(3*t*)	31	11.62	1.65	0.605
7	18:1/16:0	32	12.94	1.84	0.543
8	16:0/16:0	32	13.45	1.91	0.523
9	18:0/16:1(3*t*)	33	15.35	2.18	0.458
10	18:0/16:0	34	18.47	2.63	0.381

[a]Peak numbers correspond to those indicated in Figure 1.
[b]A new equivalent carbon number (ECN*) value has been defined (see text).
[c]The relative retention time (RRT) was calculated in terms of the retention time of the first eluted component [i.e., 18:3/16:1(3*t*)]. See Table 1 for other abbreviations.

18:n/16:1(3*t*) and 18:n/16:0 where $0 \leq n \leq 3$. In each series, the characteristics of the molecular species are determined by the number of double bonds of the C_{18} -fatty acid at the *sn*-1 position. Figure 2B shows that for each of the above molecular species series, the reciprocal of the RRT and the number of double bonds in the two C_{18} series were linearly correlated, with a correlation coefficient (r) of 1. The general equation of the straight line is $1/RRT = an + b$, where n is the number of double bonds in the C_{18}-fatty acid series, b the reciprocal of the relative retention time of 18:0/16:1(3*t*) in the 18:n/16:1(3*t*) series and of 18:0/16:0 in the 18:n/16:0 series, and a the slope of the straight line. Based on the data shown in Table 2, one can calculate the equation of each straight line:

$$\text{for the 18:n/16:1(3}t\text{) series: } 1/RRT = 0.181n + 0.456 \quad [1]$$

$$\text{for the 18:n/16:0 series: } 1/RRT = 0.162n + 0.381 \quad [2]$$

The slope (a) of the straight line characterizing the 16:1(3*t*)-containing series is greater than that of the 16:0-containing series. This indicates that the two lines are not parallel and that the presence of a *trans*-double bond at the *sn*-2 position resulted in the decrease of the RRT of the corresponding DNB derivative molecular species in the HPLC reversed-phase column (see Fig. 2A). This observation suggests that the interactions of 16:1(3*t*) or 16:0 at the *sn*-2 with the other fatty acid partners (e.g., C_{18}:*n*) of a same PG molecular species toward the stationary phase of the HPLC column are different. Thus, different relative interactions between the double bonds may explain why two molecular species having the same ECN* value but a different number of double bonds can be easily separated in the HPLC reversed column, e.g., 18:1/16:1(3*t*) and 16:0/16:1(3*t*) as well as 18:1/16:0 and 16:0/16:0 (Table 2). In addition, two molecular species having the same ECN* value and the same double-bond number but a different distribution of the double bonds in the *sn*-1 and *sn*-2 position can also be separated in the HPLC column, e.g., the 18:2/18:2 and 18:3/18:1 molecular species of phosphatidylcholine (data not shown). The former species had a RRT smaller than that of the latter species. Thus, Figure 2B allows one to predict with great accuracy the composition of any PG molecular species having 18:n/16:0 or 18:n/16:1(3*t*) configuration ($0 \leq n \leq 3$).

Verification of the validity of the method. The above method which allows the separation and identification of 3,5-DNB derivatives of PG molecular species requires two critical steps. First, the removal of the polar head group (phosphoglycerol) by the hydrolysis of PG in the presence of PLC has to be complete. Figure 3 shows that with increasing concentrations of PLC there was a concomitant decrease in PG and an increase in DAG content up to a concentration of 160 mU of PLC/about 70 nmol of PG. Beyond this minimal concentration, the hydrolysis reaction was complete. In the method described, we propose to use 1 to 10 units of PLC/70 nmol PG. In comparison, Kito *et al.* (13) have reported that 200 units of PLC/0.5 mg (about 700 nmol) phosphatidylcholine were required in spite of the fact that phosphatidylcholine is a better substrate than PG for the PLC from *B. cereus* (21). Second, the transformation of the resulting DAG molecules into 3,5-DNB DAG also has to be complete without any modification of the fatty acid composition. Kito *et al.* (13) have demonstrated that under conditions identical to ours, 98.8% of the DAG are converted to 3,5-DNB–DAG. Furthermore, we have verified that the products of the enzymatic (DAG) and chemical (DNB–DAG) reactions have the same fatty acid composition as that of the initial phospholipid (PG) as illustrated in Figure 4.

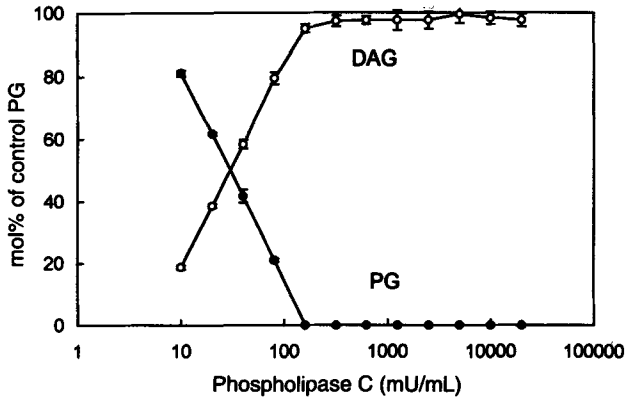


FIG. 3. Dependence of the formation of diacylglycerol (DAG; ○) from phosphatidylglycerol (PG; ●) on the concentration of phospholipase C. The results are presented as means ± standard deviation of triplicate samples.

Finally, it was necessary to verify that the areas of the HPLC peaks obtained at 254 nm are not affected by the differences in the structure of the PG molecular species. To this end, five samples of DNB–DAG corresponding to 2.5 (12.5 μg chlorophyll), 5 (25 μg), 10 (50 μg), 20 (100 μg), and 40 (200 μg) nmol were injected in the HPLC column. Figure 5

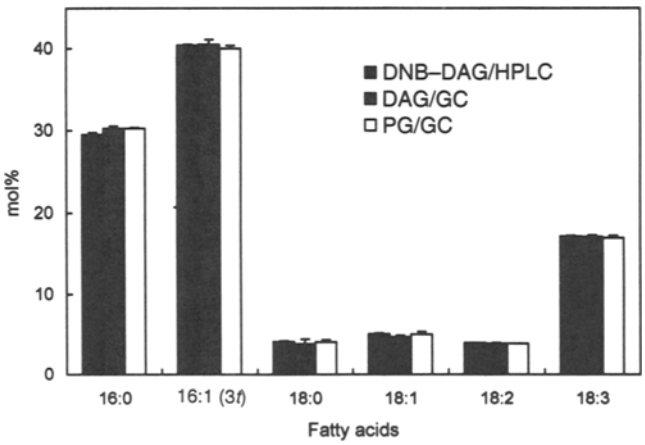


FIG. 4. Comparison of the fatty acid composition of PG, DAG obtained after phospholipase C treatment, and 3,5-dinitrobenzoyl–diacylglycerol (DNB–DAG) molecules obtained after derivatization. The fatty acid content in PG and DAG was measured by gas chromatography as described in the Experimental Procedures section. The fatty acid composition of DNB–DAG was calculated on the basis of the molecular species obtained by high-performance liquid chromatography (HPLC) (see Table 1). See Figure 3 for other abbreviations.

shows that the amount of DNB–DAG injected in the HPLC was linearly correlated with the peak area. This was true for the ten PG molecular species encountered in the thylakoid membranes of squash. From these data and those reported in Table 1, one can calculate the range limits within which the peak areas were proportional to the amounts of DNB–DAG. The upper limit (see arrow in Fig. 5) was found for the DNB–DAG [16:0/16:1(3*t*)] which corresponded to about 12 nmol (i.e., 40 nmol of DNB–DAG injected × 31 mol% of total PG/100 = 12.4 nmol). The lower limit (see arrow in Fig. 5) was obtained for the DNB–DAG (18:0/16:0) which corresponded to 0.025 nmol (i.e., 2.5 nmol of DNB–DAG injected × 1 mol% of total PG/100 = 0.025 nmol). Though not shown in Figure 5, the detection limit of DNB–DAG (18:0/16:0) can be lowered to 6 pmol, an amount which is generally sufficient for characterizing the PG molecular species in higher plants. When higher sensitivities are needed, new techniques such as electrospray mass spectrometry (22) or HPLC/mass spectrometry using electrospray ionization (23) allow the quantification of phospholipid molecular species as low as approximately 0.5 pmol (23).

Conclusions. The fact exists that PG molecular species encountered in photosynthetic membranes from higher plants contain, in addition to *cis*-double-bond fatty acids, one *trans*-double bond in 16:1(3*t*), required a new theoretical approach on the relationship between the HPLC experimental data (e.g., retention time or its reciprocal) and the structural characteristics of each molecular species (carbon and double-bond number). A new ECN value $ECN^* = CN - 2n_c - n_t$, where CN corresponds to the number of carbon atoms in the aliphatic residues and n_c and n_t to the number of *cis*- and *trans*-double bond per molecular species. Moreover, the ten PG molecular species, except 16:0/16:0 and 16:0/16:1(3*t*), fit into the two series: 18:n/16:1(3*t*) and 18:n/16:0 ($0 \leq n \leq 3$) where the characteristics of the molecular species are determined by the number of double bonds of the C_{18}-fatty acid at the *sn*-1 position. The reciprocal of the retention time and the number of double bonds in the two C_{18} series are linearly correlated (r = 1).

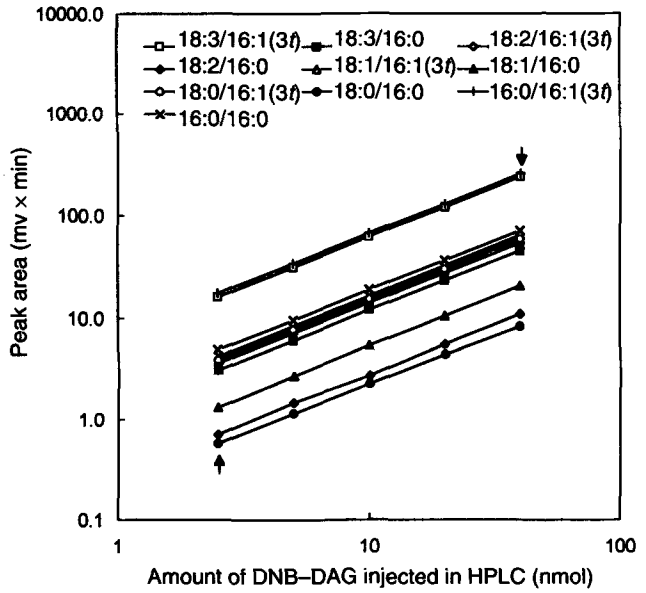


FIG. 5. Proportionality between the amounts of DNB–DAG injected in the HPLC column and the peak areas of each DNB–DAG molecular species. For further explanation, see the text. See Figures 3 and 4 for abbreviations.

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The Use of Nicotinates and Sulfoquinovosyl Monoacylglycerols in the Analysis of Monounsaturated n-3 Fatty Acids by Mass Spectrometry

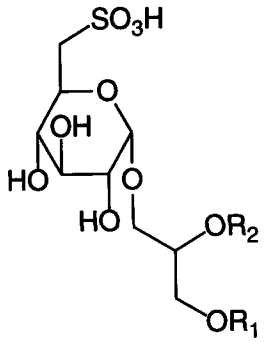
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ABSTRACT: Fatty acyl groups (16:1 and 16:0) liberated from purified sulfoquinovosyl diacylglycerols produced by the unicellular marine microalga, *Heterosigma carterae* (formerly *H. akashiwo*), were converted to either the corresponding alcohols or methyl esters. Nicotinate derivatives of the alcohols were examined by combined gas chromatography/mass spectrometry, and the methyl esters were examined by nuclear magnetic resonance (NMR) spectroscopy after separation by high-performance liquid chromatography. Three different hexadecenoyl fatty acyl groups were identified, one of which was *cis* 13-hexadecenoyl (16:1n-3). Both the configuration and the n-3 position of the double bond in the *cis* 13-hexadecenoyl moiety were unequivocally established by NMR analysis of the corresponding methyl ester. The nicotinate derived from the alcohol of the 16:1n-3 fatty acyl moiety gave a characteristic fragmentation series in the electron impact mass spectrum which, by careful interpretation, was consistent with, but not unambiguous for, the assigned location of the double bond. Tandem mass spectrometry experiments on a sulfoquinovosyl monoacylglycerol containing the *cis* 13-hexadecenoyl group in the *sn*-2 position, using negative-ion liquid secondary ion mass spectrometry, also gave a fragmentation pattern which was consistent with the positional assignment of the double bond.

Lipids 31, 231–238 (1996).

Due to their activity against the human immunodeficiency virus, sulfoquinovosyl diacylglycerols (SQDG) have attracted considerable attention in the past few years (1). SQDG are widely distributed in nature, occurring in most higher plants, algae, and in a purple nonsulfur bacterium. Because of the difficulties in separation and purification of the naturally-occurring mixtures of SQDG (for a general structure see Scheme 1), which differ only in the length and/or degree of unsaturation of the fatty acyl substituents, a practical and rapid method for the structural analysis of these substances has been elusive (2). As part of a program to develop such a general method, we have examined the complex mixture of unusual SQDG produced by *Heterosigma carterae* (formerly *H. akashiwo*) (3), a fish-killing microalga (4). One of the acyl groups liberated from the SQDG produced by *H. carterae* was identified as *cis* 13-hexadecenoyl, the structure determination of which is reported herein.



SCHEME 1

In the past, 13-hexadecenoic acid (16:1n-3) has been identified in lipids by gas chromatographic techniques (5–7). However, the fatty acid structural assignments were not always confirmed by nuclear magnetic resonance (NMR) techniques, nor by gas chromatography/mass spectrometry (GC/MS) experiments (i.e., preparation and analysis of suitable derivatives which could confirm the assumed structure by a specific fragmentation). In recent years, picolinyl ester derivatives of fatty acids and nicotinates of long-chain alcohols were found to be suitable for the positional determination of double bonds by mass spectrometry, and were tested for a number of standard compounds (8–12). However, because monounsaturated n-3 fatty acids or the analogous alcohols are not commercially available, these derivatization techniques have not been fully evaluated for such compounds (12). After submission of this report, it came to our attention that *cis* 15-octadecenoic acid (18:1n-3) is now available from Sigma Chemical Company (St. Louis, MO). In preliminary experiments, it was found that the fragmentation pattern in the electron impact (EI) mass spectrum of the nicotinate ester derivative of one of the three SQDG 16:1 acyl groups pro-

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Abbreviations: COSY, correlated spectroscopy; EI, electron impact; GC/MS, gas chromatography/mass spectrometry; LSIMS, liquid secondary ion mass spectrometry; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; SQDG, sulfoquinovosyl diacylglycerol; SQMG, sulfoquinovosyl monoacylglycerol; TLC, thin-layer chromatography.

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duced by *H. carterae* was ambiguous with respect to the double bond position (either n-3 or n-4). Furthermore, due to the lack of commercially-available standard fatty acids and alcohols, it was not possible to make the distinction between n-3 and n-4 monounsaturated nicotinates on the basis of their gas chromatography (GC) retention times. Thus, a simple interpretation of the EI mass spectra of nicotinate derivatives of n-3 and n-4 monounsaturated fatty alcohols may result in the wrong assignment—a problem which, to our knowledge, has not been previously reported. To overcome this problem, we have investigated, in detail, the fragmentation pattern for the nicotinate derivative of the *cis* 13-hexadecenoyl moiety liberated from *H. carterae* SQDG, and, more importantly, we unequivocally established the n-3 position of the double bond by NMR analysis of the corresponding methyl ester.

We have also utilized the sulfoquinovosyl moiety as a "natural derivative," which acts by localizing the negative charge at the sulfonic acid moiety, and hence might be suitable for the assignment of the double bond position in the attached fatty acyl group by MS. Although SQDG can potentially yield the required information *via* negative-ion tandem mass spectrometry (MS/MS), in experiments using liquid secondary ion mass spectrometry (LSIMS) ionization, the interpretation of the spectra in terms of the double bond positions is difficult because there are two fatty acyl chains involved in the SQDG structure (13). For this reason, an SQDG which contained the *cis* 13-hexadecenoyl moiety in the *sn*-2 position was converted to the corresponding sulfoquinovosyl monoacylglycerol (SQMG) by an enzymatic reaction (lipase type XI, specific for hydrolysis the *sn*-1 acyl moiety). The negative-ion LSIMS MS/MS spectrum of the resulting SQMG was compared with that of an SQMG containing the hexadecanoyl group in the *sn*-2 position. This latter substance was derived from SQDG isolated from the brown macroalga *Alaria esculenta*. It was shown that SQMG are suitable for the assignment of the double bond position in n-3 fatty acids.

MATERIALS AND METHODS

Extraction and isolation of (SQDG) from H. carterae *and* A. esculenta. Wet cell material of *H. carterae* (160 g, strain 102-R, cultivated in natural sea water) was extracted with 1 L of MeOH/$CHCl_3$ (7:3, vol/vol) for 30 min at 7°C. The mixture was bubbled with nitrogen and sonicated. The extract was filtered, and the residue was washed three times with 150 mL of MeOH/$CHCl_3$ (7:3, vol/vol). The combined filtrates were evaporated to dryness under reduced pressure to yield 8.4 g of crude extract. The extract was loaded on a silica gel column (30 mm i.d., 100 g silica gel) and the SQDG were eluted using a gradient consisting of the following steps: (i) 200 mL *n*-hexane/$CHCl_3$ (2:1, vol/vol); (ii) 250 mL $CHCl_3$; (iii) 250 mL $CHCl_3$/MeOH (3:1, vol/vol); (iv) 250 mL $CHCl_3$/MeOH (1:1, vol/vol); and (v) 500 mL MeOH. The separation was monitored by thin-layer chromatography (TLC). A total of 850 mg SQDG was isolated. The ^1H and ^{13}C NMR spectra of this mixture of SQDG were in accordance with published results (1,14).

The crude SQDG fraction was separated on a Nova-Pak C_{18} high-performance liquid chromatography column (7.8 mm i.d. × 300 mm; Waters Limited, Milford, MA) using a linear gradient from MeOH/H_2O (7:3, vol/vol) to 100% MeOH (3 mL/min; two Waters M6000 pumps combined with a gradient controller; Ultraviolet-detection at 215 nm with a Waters 484 UV-detector) to give 101 mg of SQDG, which contained the 16:1/16:0 fatty acyl pair (retention volume 113 mL). The identities of these fatty acyl moieties were confirmed by LSIMS. The resultant LSIMS MS/MS fragmentation was in accordance with published results (13).

The brown alga *A. esculenta* (275 g wet weight) was collected at Herring Cove near Halifax, Nova Scotia, Canada, in June 1994. Immediately after collection, the tissues were frozen in liquid nitrogen, ground in a blender in a frozen state, and then extracted with 1 L of MeOH/$CHCl_3$ (7:3, vol/vol). The crude extract was fractionated in the same way as described previously to yield 360 mg of a mixture of SQDG.

Reductive cleavage of SQDG (16:1/16:0 fatty acyl pair) from H. carterae: *Preparation of alcohols.* One hundred milligrams of $LiAlH_4$ were carefully added to diethyl ether (3 mL) and stirred until hydrogen evolution stopped. A solution of 15 mg SQDG (16:1/16:0 fatty acyl pair) in 1,4-dioxane (0.5 mL) was then added, and the mixture was stirred for 30 min under reflux. After cooling, the reaction mixture was carefully added to 10 mL ice-cold deionized water. The water layer was acidified to pH 1 with dilute sulfuric acid and then extracted five times with 20 mL of hexane. The combined hexane layers were dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure. The resulting colorless oil (5 mg) was examined by TLC (Merck Kieselgel 60 F_{254}, 0.25 mm layer; Merck, Darmstadt, Germany) and ^1H NMR (Bruker AMX 500; Bruker, Ontario, Canada). The TLC plates were developed with *n*-hexane/diethyl ether (3:1, vol/vol). After dipping in *p*-anisaldehyde/sulfuric acid/acetic acid/ethanol (2.7:3.3:1.1:93.0, by vol), and heating the plate, a single blue spot at $R_f = 0.21$ was observed. The ^1H NMR spectrum ($CDCl_3$) of this material shows, in addition to the typical signals for long-chain alkyl groups, a methylene triplet at δ 3.62 ppm, indicative of the newly formed mixture of 16:1/16:0 alcohols.

Conversion of nicotinic acid to the acid chloride. Two hundred milligrams of nicotinic acid (BDH Chemicals Ltd., Poole, Dorset, United Kingdom) and 0.5 mL of SO_2Cl_2 (Aldrich Chemical Co., Inc., Milwaukee, WI) were combined in a reaction vial and tightly sealed. The mixture was heated to 100°C for 10 min. After cooling, the remaining SO_2Cl_2 was removed with a stream of nitrogen. The final product formed colorless needles and sublimed easily. It was found to be suitable for the esterification reaction without recrystallization or sublimation.

Synthesis of the nicotinates. This procedure is based on the synthesis developed by Vetter and Meister (11). The mixture of 16:1 and 16:0 long-chain primary alcohols (5 mg) were dissolved in 0.5 mL anhydrous pyridine (Aldrich Chemical Co., Inc.) in a reaction vial. Nicotinyl chloride hydrochloride

(25 mg) was added and, after flushing with nitrogen, the vial was tightly sealed. The mixture was stirred at 100°C for 10 min. The resulting clear, slightly yellow solution was then added to an aqueous saturated NaCl solution (10 mL), and extracted five times with 10 mL of *n*-hexane. The combined hexane layers were dried over anhydrous Na_2SO_4. After filtration, the solvent was evaporated under reduced pressure to give 3 mg of a slightly yellow oil which was examined by ^1H NMR ($CDCl_3$). The methylene triplet (δ 3.62 ppm) had now shifted downfield to δ 4.37 ppm, indicating the successful formation of the nicotinate ester derivatives. The pyridine moieties of the formed nicotinates showed signals at δ 7.54, 8.45, 8.81, and 9.25 ppm. The crude mixture of 16:1 and 16:0 nicotinates was directly analyzed by GC/MS without further purification. The nicotinate derivatives of 16:1n-3 (100%), 16:1n-5 (20%), 16:1n-7 (20%), and 16:0 (130%) fatty alcohols were detected (ratios were determined from the total ion chromatogram peak areas, with the peak area for the 16:1n-3 nicotinate normalized to 100%). The individual nicotinates were not isolated.

Enzymatic cleavage of SQDG (16:1n-3/16:0 fatty acyl pair): Preparation of 16:1n-3 SQMG. The enzymatic digestion is based on the procedure described by Fischer, *et al.* (15). A portion of the SQDG sample (75 mg) containing the 16:1/16:0 fatty acyl pair isolated from *H. carterae* was separated into seven fractions on the Nova-Pak C_{18} column using a 30-min, 3 mL/min gradient consisting of MeOH/H_2O (9:1, vol/vol) isocratic for 5 min, then a linear increase to 9.4:0.6 MeOH/H_2O (vol/vol) over 15 min, then a linear increase to 9.5:0.5 (vol/vol) over 5 min, followed by a linear gradient over 5 min to 100% MeOH, and monitoring absorption at 215 nm. The fraction highly enriched in the SQDG with the 16:1n-3/16:0 fatty acyl pair (retention volume 62.1 mL) was identified by ^1H and ^{13}C NMR analysis, including ^1H–^1H COSY, heteronuclear multiple quantum correlation, and heteronuclear multiple bond correlation. A total of 3 mg of this fraction was dissolved in MeOH (0.5 mL), containing 1 mg of Triton X-100 (Union Carbide, Toronto, Ontario, Canada). The solvent was removed under a stream of nitrogen. The residue was dissolved in Tris buffer (0.5 mL, pH 7.4 at room temperature), and 5 mg of lipase solution (lipase type XI from *Rhizopus arrhizus*; Sigma Chemical Co., St. Louis, MO) was added. The mixture was stirred at 37°C for 15 min. The reaction was stopped by adding isopropanol (15 mL), and the solvent was evaporated to dryness under reduced pressure. The residue was loaded onto a flash column (8 mm i.d.) packed with 2.5 g of C_{18} derivatized silica gel (Baker, Toronto, Ontario, Canada) and eluted with water (25 mL) followed by MeOH (25 mL). The methanol fraction containing the 16:1n-3 SQMG was analyzed by LSIMS MS/MS.

Enzymatic cleavage of SQDG from A. esculenta: *Preparation of SQMG.* The crude mixture (3 mg) of SQDGs from *A. esculenta* was hydrolyzed in the same manner as described previously. The methanol fraction containing the SQMG was analyzed by LSIMS MS/MS.

SQDG: Preparation of fatty acid methyl esters. Fifteen

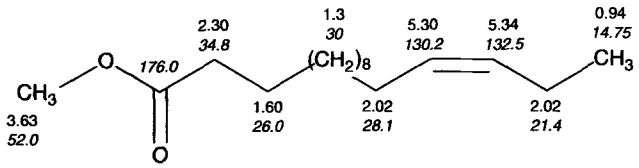


FIG. 1. Proton and ^{13}C assignments for *cis* 13-hexadecenoic acid methyl ester (CD_3OD). ^{13}C chemical shifts are in *italics*. The nuclear magnetic resonance (NMR) assignments were confirmed by two dimensional NMR experiments.

milligrams of the SQDG (16:1/16:0 fatty acyl pair) isolated from *H. carterae* were dissolved in absolute MeOH (5 mL) and a few drops of trifluoroacetic acid were added. After 5 min, the solvent was evaporated under reduced pressure, and the mixture was separated on the Nova-Pak C_{18} column using a 20 min linear gradient system of acetonitrile/H_2O (7:3, vol/vol) to 100% acetonitrile (3 mL/min), and compound detection was done at 215 and 230 nm with a Waters 994 diode array detector (the ratio of the peak intensities between 215 and 230 nm yields some information about the degree of unsaturation in the fatty acid methyl esters). A total of 2.2 mg of hexadecenoic acid methyl esters, comprised of about 70% *cis* 13-hexadecenoic acid methyl ester, was isolated (retention volume 81.6 mL). Further purification of this material by separation on a Zorbax R_X-C18 column (10 mm i.d. × 250 mm; MAC-MOD Analytical, Inc., Brockville, Ontario, Canada) using a linear gradient (3–17 min, 3.5 mL/min) from MeOH/H_2O (8:2, vol/vol, 0–3 min, 3.5 mL/min) to MeOH/H_2O (9:2, vol/vol, 17–30 min, 4.0 mL/min) gave 0.7 mg of *cis* 13-hexadecenoic acid methyl ester (retention volume 95 mL). The NMR assignments for this substance, obtained using COSY, heteronuclear multiple quantum correlation, and heteronuclear multiple quantum correlation and heteronuclear multiple bond correlation experiments, are given in Figure 1. The two remaining 16:1 fatty acid methyl esters were identified as the methyl esters of palmitoleic acid (*cis* 16:1n-7) and 11-hexadecenoic acid (16:1 n-5) (Keusgen, M., Curtis, J.M., and Ayer, S.W., unpublished data).

MS. All MS experiments were performed using a VG ZAB-EQ mass spectrometer (Fisons Instruments, Manchester, United Kingdom) controlled by the VG Opus software. All GC/MS experiments were done with a Hewlett-Packard (Avondale, PA) 5890 Series II gas chromatograph and on-column injector. A 30-m DB225 capillary column (J&W Scientific, Folsom, CA) with an i.d. of 0.25 mm and a film thickness of 0.25 μm was used. The gas chromatograph was operated in constant flow mode at a rate of 50 cm/s of He carrier gas. The temperature program was as follows: initial temperature 70°C for 1 min, ramp at 20°C/min to 180°C, ramp at 3°C/min to 240°C, and oven hold for 20 min. EI experiments were performed at an ion source temperature of 200°C and an ionizing electron energy of 70 eV. LSIMS experiments used a 20 keV beam of Cs^+ ions and a matrix of triethanolamine. MS/MS experiments were performed in the first field-free region by a linked scan at constant B/E using Ar collision gas

at a pressure which attenuated the ion beam by 50% and at a collision energy of 8 keV.

RESULTS AND DISCUSSION

Vetter and Meister (11) first described the use of nicotinates as derivatives for the structure elucidation of long-chain alcohols. Induced by a hydrogen transfer to the pyridine nitrogen, a fairly regular fragmentation series results due to remote-site radical-induced cleavages along the chain. Harvey (10) described a very similar fragmentation for picolinyl esters obtained from fatty acids. For the investigation of SQDG fatty acyl groups, reductive cleavage of the ester bonds and conversion of the formed alcohols into nicotinates was found to be the best procedure [use of the picolinyl ester methodology gave poor recovery of the polyunsaturated fatty acid derivatives (Keusgen, M., Curtis, J.M., and Ayer, S.W., manuscript in preparation)]. A major alcohol liberated in the investigated SQDG fraction from *H. carterae* was identified as *cis* 13-hexadecenol, as described later. The corresponding fatty acid is *cis* 13-hexadecenoic acid (*cis* 16:1n-3). The n-3 position and *cis* configuration of the carbon–carbon double bond were determined by NMR analysis of the methyl ester of this acid. In a ^{1}H NMR decoupling experiment, irradiation of the multiplet at δ 2.02 ppm (CD_3OD) furnished an AB spin system for the olefinic protons, with a coupling constant of J_{AB} = 10.8 Hz, a value typical for olefinic protons in the *cis* configuration (16). In the same experiment, the signal for the terminal methyl group collapsed to a singlet, a result which allowed unambiguous assignment of the n-3 position for the double bond. Additionally, the ^{1}H–^{1}H COSY spectrum of this methyl ester (Fig. 2) clearly shows cross peaks for correlation of the resonance for the terminal methyl group (δ 0.94 ppm) with the allylic protons resonating at δ 2.02 ppm. The ^{13}C NMR spectrum shows signals at δ 130.2 and 132.5 ppm, which are also characteristic for olefinic carbons in monounsaturated fatty acid methyl esters bearing olefinic protons in the *cis* configuration (Fig. 1) (17,18).

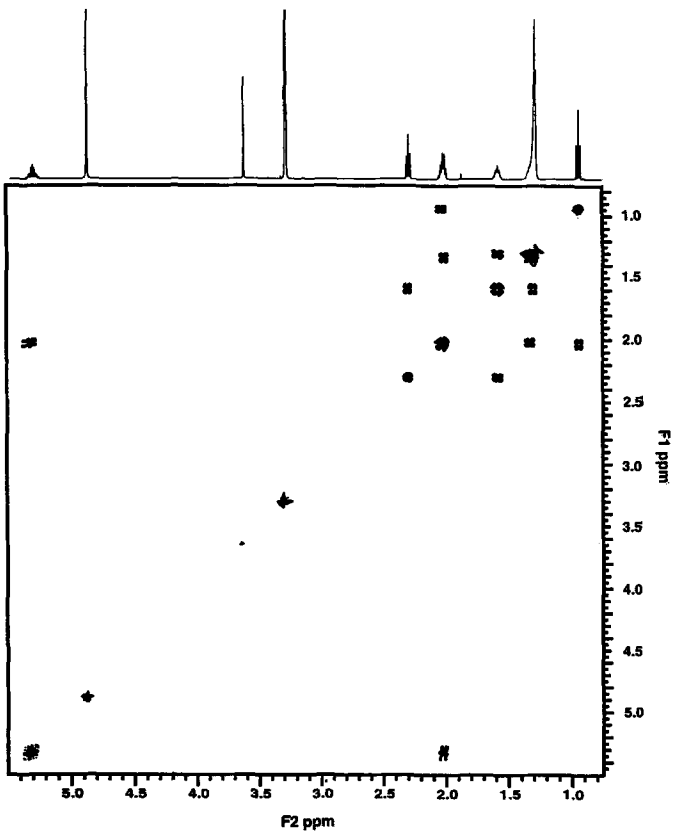


FIG. 2. Proton–proton COSY nuclear magnetic resonance (NMR) spectrum of *cis* 13-hexadecenoic acid methyl ester (CD_3OD) with the one-dimensional proton NMR spectrum positioned at the top of the COSY spectrum.

For the determination of the double bond position in monounsaturated fatty acids, a direct comparison between the derivatives of a saturated and monounsaturated fatty acids with the same number of carbons is helpful (e.g., 16:0, 16:1n-7, and 16:1n-3). Figure 3 shows mass spectra of the nicotinate esters of hexadecanol (Fig. 3A) and *cis* 9-hexadecenol (Fig. 3B), and Figure 4 shows the mass spectrum for the nicotinate ester of *cis* 13-hexadecenol. These nicotinates were resolved by GC (retention times of 22.6, 23.4, and 24.0 min, respectively), and were obtained after liberating the corresponding alcohols from the SQDG purified from extracts of *H. carterae*. For the nicotinate ester of hexadecanol, an ion at *m/z* 332, due to the loss of the terminal methyl group ($[M–CH_3^•]^+$), and an additional fragment ion at *m/z* 318, resulting from the loss of the terminal ethyl group ($[M–CH_3CH_2^•]^+$), are clearly visible. The spectrum also shows a remarkably distinct series of CH_2 losses from *m/z* 318 to 178, arising from remote-site radical-induced cleavages further along the alkyl chain. Note that the fragment ion at *m/z* 332 has a significantly lower intensity than the following ones (*m/z* 318 to 178). Below *m/z* 178, rearrangement processes reflecting hydrogen transfer onto the pyridine ring dominate. This results in the ions at *m/z* 165, 151, and 137 rather than at one amu lower as would be expected for simple remote-site radical-induced cleavages. The base peak ion at *m/z* 124 is characteristic for the nicotinates. Its formation can be rationalized by transfer of two hydrogen atoms and loss of the alkyl chain to give the *N*-protonated nicotinic acid (11).

It is well known that when the alkyl chain also contains a double bond, this pattern of peak intensities is changed (10–12). The published spectra of picolinyl and nicotinate ester derivatives of monounsaturated fatty acids and long chain alcohols, respectively [e.g., *cis* 9-octadecenoic acid (10) and *cis* 9-octadecenol (11)], clearly show that the fragment ions arising from the formal cleavage of the double bond (e.g., between a and a′, Fig. 3B) or vinylic cleavages (e.g., between a and b, or between a′ and b′, Fig. 3B) give weak signals. In contrast, peaks representing cleavages at the allylic positions (e.g., between b and c, or between b′ and c′, Fig. 3B) are clearly visible in the published spectra. In addition, the fragment ions resulting from cleavage on the distal side of the double bond (e.g., between b′ and c′, or c′ and d′, Fig. 3B), are significantly enhanced in intensity (10–12).

When there is a double bond in the n-3 position, as for the

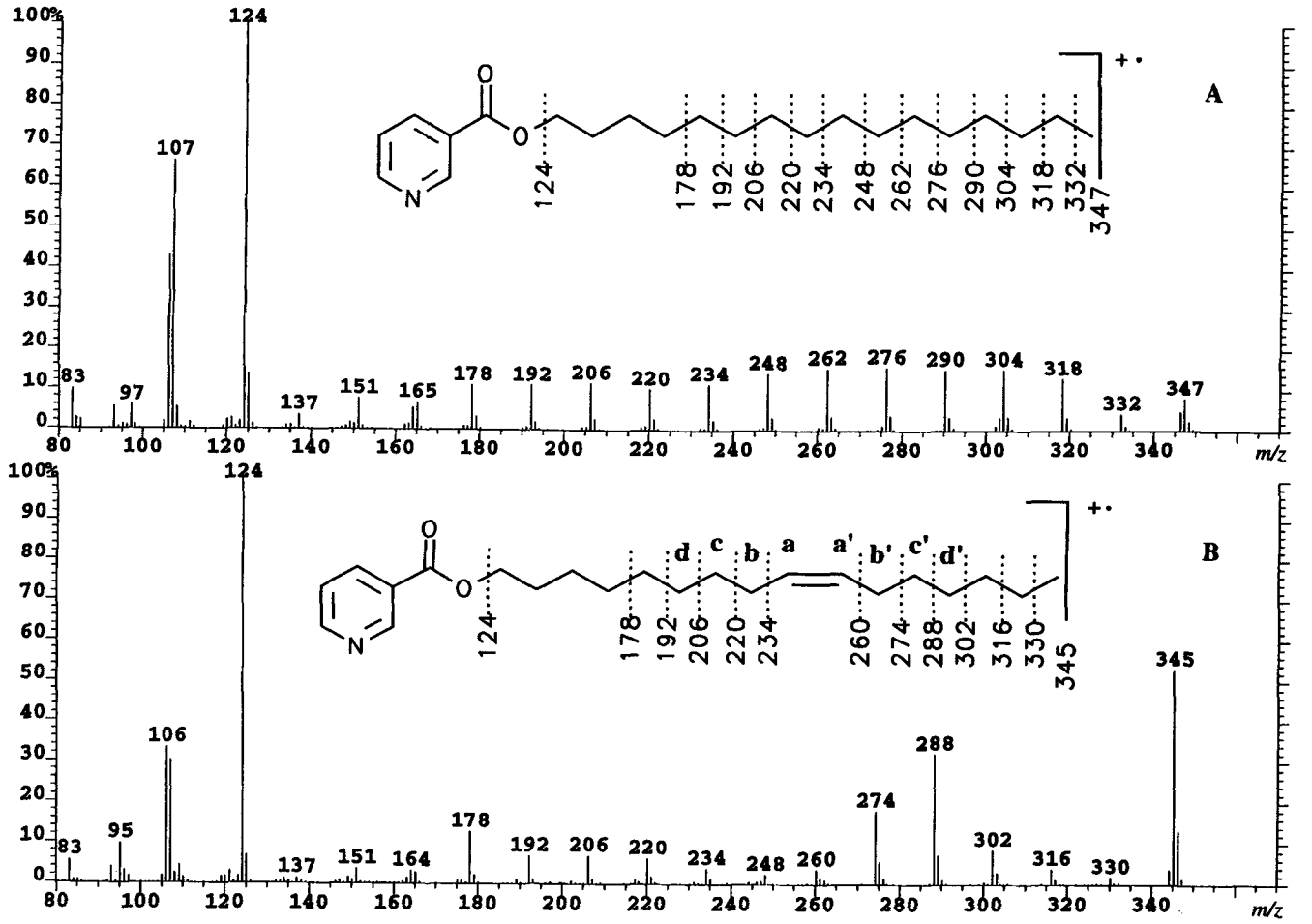


FIG. 3. The gas chromatography/mass spectrometry election impact mass spectra of the nicotinate derivatives obtained from (A) hexadecanol, and (B) *cis* 9-hexadecenol, by reductive cleavage of a sulfoquinovosyl monoacylglycerol followed by esterification.

nicotinate derivative of *cis* 13-hexadecenol, the results are less clear (Fig. 4). In this case, there are two superimposed effects on the relative fragment ion intensities which must be considered:—(i) for saturated alkyl chains, a relatively weak signal results from the loss of the terminal methyl group, while the signal coming from the loss of the terminal ethyl group is much stronger; and (ii) the fragment intensities resulting from cleavages at the isolated double bond, as described previously. From interpretation of the spectrum shown in Figure 4 based on literature precedent (8–11), and in comparison to Figure 3B, it would appear that the double bond is located at the n-4 position. Formal cleavage of the double bond would correspond to the low relative intensity cluster of fragment ions at *m/z* 290–288, and the relatively intense fragment ion at *m/z* 302 would correspond to a distal vinylic cleavage. In the present case, however, the nicotinate derivative is known to be that of an n-3 monounsaturated fatty alcohol (i.e., by interpretation of the NMR data for the *H. carterae* SQDG 16:1n-3 fatty acyl methyl ester derivative), and the correct interpretation could possibly be made by considering the regular fragmentation series from *m/z* 276–178, and the relative intensities of the *m/z* 330 and 316 fragment ions. If the double bond had been located in the n-4 position, the peak at *m/z* 276 would be expected to have a significantly weaker intensity than the following ones (*m/z* 262—178), as it would represent cleavage at a vinylic position. Additionally, Figure 4 shows that the peak at *m/z* 330, which represents the loss of the terminal methyl group, has nearly the same intensity as the peaks between *m/z* 276 and 178 (compared with the relative intensities of the analogous fragment ions at *m/z* 330, and between *m/z* 220 and 178, for the nicotinate derivative of the 16:1n-7 SQDG acyl moiety as shown in Fig. 3B). This enhanced relative intensity can be explained by the fact that the terminal methyl group is in an allylic position. In an analogous way, the fragment ion at *m/z* 274 in Figure 3B, which represents cleavage at the distal allylic position for the nicotinate ester of the 16:1n-7 SQDG fatty acyl group, also shows enhanced intensity. The signal at *m/z* 316 in Figure 4 represents a formal cleavage at the distal vinylic position. This type of fragmentation usually produces weak signals, but, in the case of the nicotinate ester of *cis* 13-hexadecenol, this signal has a high relative intensity, a result which is consistent with the EI mass spectral data reported for the picolinyl ester derivative of *cis* 15-octadecenoic acid

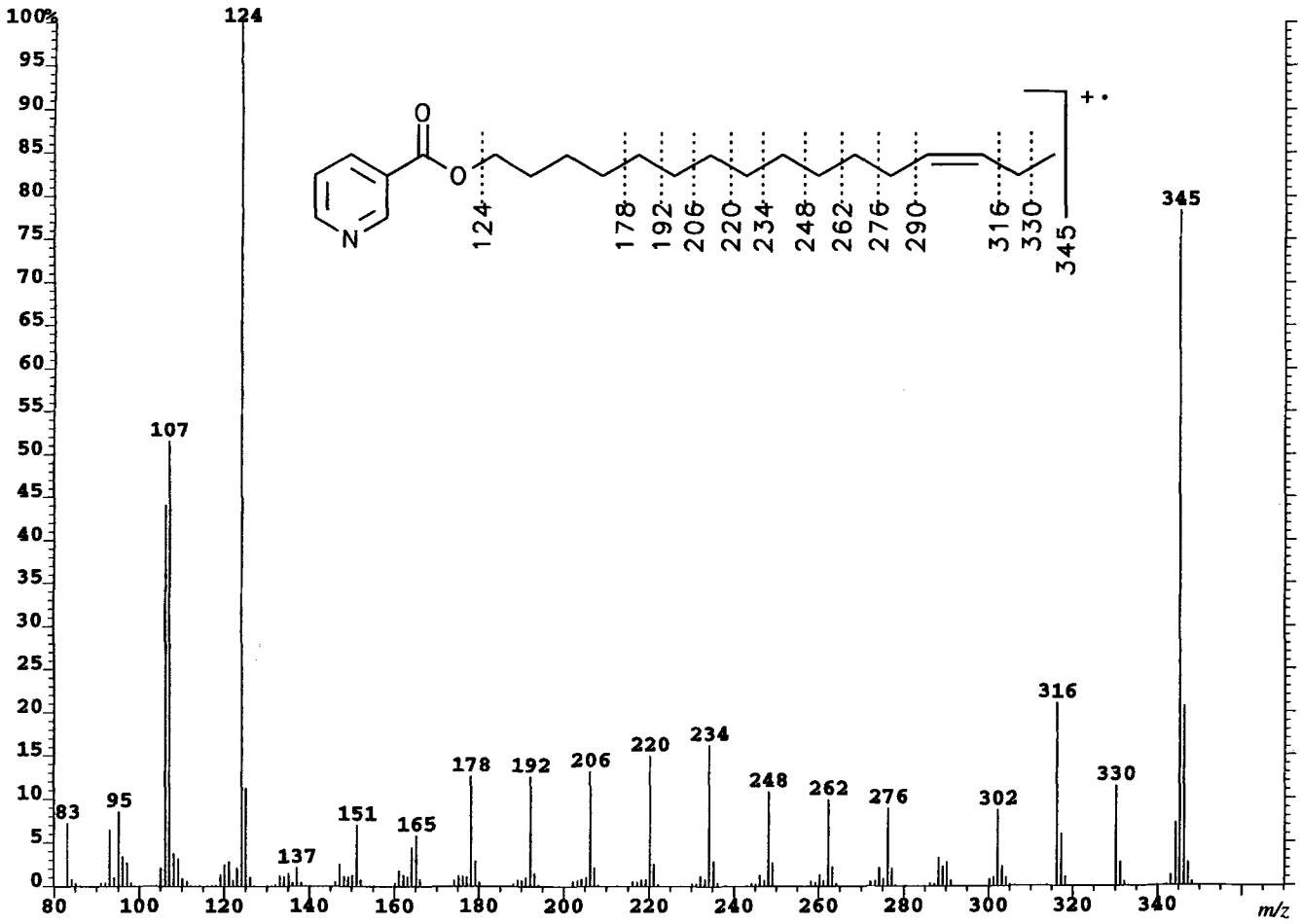


FIG. 4. The gas chromatography/mass spectrometry election impact mass spectrum of the nicotinate derivative obtained from *cis* 13-hexadecenol by reductive cleavage of a sulfoquinovosyl monoacylglycerol followed by esterification.

(18:1n-3) (12). The intensity of the fragment ion representing the distal vinylic cleavage may be expected to be weak for nicotinates with the double bond further up the chain. The ambiguous ions at *m/z* 290 or 288 are interpreted as arising as a result of cleavage at the interior vinylic position (expected to be of low relative intensity), and the ion at *m/z* 302 is interpreted as arising from cleavage of the double bond. The relative intensity of *m/z* 302 ion is certainly anomalous. In results for picolinyl ester and nicotinate derivatives of fatty acids which have been fully reported to date, the relative abundance of fragment ions resulting from cleavage of the double bond are usually the smallest of any neighboring fragment ions (8–11).

In summary, for the determination of the double bond location in nicotinates derived from monounsaturated fatty alcohols, it is essential to consider the relative peak intensities, in addition to the actual *m/z* values of the fragment ions. The identification of a mass difference of 26 amu, without considering the peak intensities, is not sufficient to unambiguously locate the carbon–carbon double bond position in the nicotinate ester of *cis* 13-hexadecenol. Of course, the ideal experiment would be comparison with the nicotinate of a standard fatty alcohol of the expected structure but, as stated previously, in the case of n-3 monounsaturated fatty acids and alcohols, these compounds are not commercially available. After submission of this report, it came to our attention that *cis* 15-octadecenoic acid (18:1n-3) is now available from Sigma Chemical Company (St. Louis, MO).

We have also evaluated an alternative approach to the identification of the n-3 unsaturated fatty acyl groups present in the SQDG isolated from *H. carterae*. Fischer *et al.* (15) described a reaction with a lipase produced by *Rhizopus arrhizus* which specifically catalyzes the removal of the fatty acid at the *sn*-1 position of SQDG to yield an SQMG. The fatty acid liberated by the enzyme also can be recovered and analyzed, if this is required, to complete the structural determination of the SQDG. This selective lipase reaction was applied to the SQDG isolated from *H. carterae* which contained the *cis* 13-hexadecenoic/hexadecanoic acid pairing. The mass spectrum of the resulting SQMG, obtained by LSIMS in the negative-ion mode using a triethanolamine matrix, showed a deprotonated molecular ion at *m/z* 553, indicating that the *cis* 13-hexadecenoic acid is attached to the *sn*-2 position of the glycerol moiety. The analogous SQMG with hexadecanoic acid at the *sn*-2 position was obtained from the SQDG fraction of the brown alga *A. esculenta* (*m/z* 555 for $[M - H]^-$).

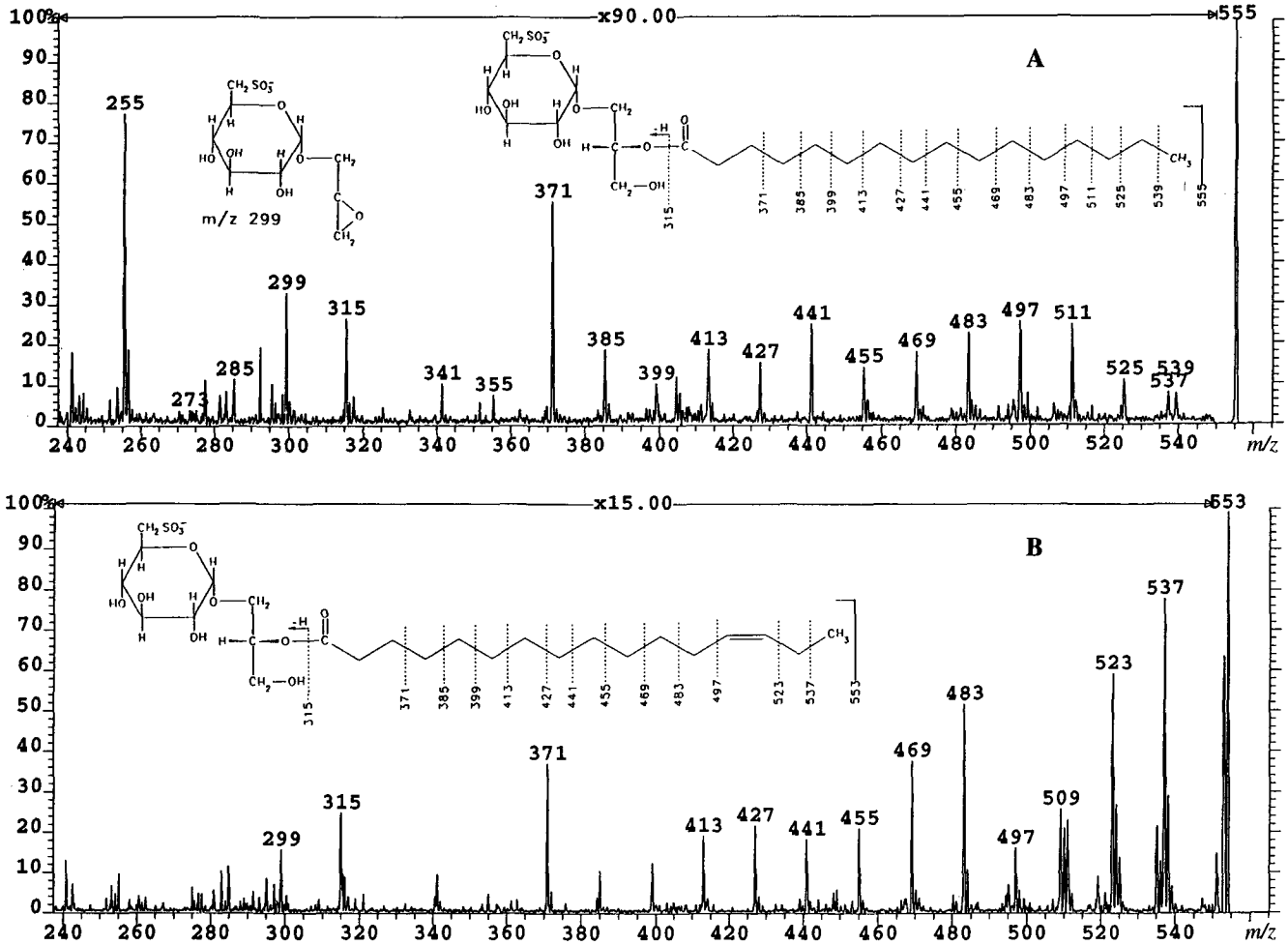


FIG. 5. The fragment ion mass spectra (liquid secondary ion tandem mass spectrometry) of a sulfoquinovosyl monoacylglycerol(SQMG) containing (A) hexadecanoyl, or (B) *cis* 13-hexadecenoyl, in the *sn*-2 position. The SQMG were obtained from the respective sulfoquinovosyl diacylglycerol by a lipase catalyzed reaction.

Because LSIMS produces little fragmentation and, hence, little structural information, it was necessary to induce fragmentation *via* collisionally-activated dissociation and record the fragment-ion spectrum (MS/MS). Due to the fact that the negative charge is localized on the sulfonic acid group, charge-remote fragmentation occurs along the alkyl chain (Fig. 5) in an analogous way to the remote-site radical-induced fragmentation observed in the positive-ion EI mass spectra of the nicotinates (Fig. 3). For SQDG, this type of fragmentation was first described by Gage *et al.* (13). However, without using the *sn*-1 deacylation, the resulting MS/MS spectra are very complex.

Figure 5A shows the negative-ion LSIMS MS/MS of the SQMG, which contains hexadecanoic acid. Starting from *m/z* 555 (the deprotonated molecular ion), a series of fragment ions is clearly visible. For a saturated fatty acyl chain this series can be explained by the loss of C_nH_{2n+2} (19,20). Thus, the loss of the terminal methyl group is manifested as a loss of CH_4 rather than $CH_3^{\bullet}$. The fragmentation of the acyl chain is fairly regular down to *m/z* 371. The spectrum also shows a significant peak at *m/z* 537, which can be explained by the formal loss of H_2O, and formation of a double bond at the glycerol backbone. Theoretically, a fragmentation series from this ion down to *m/z* 353 (signal at *m/z* 371 minus 18 amu) should be visible, but the intensities of the corresponding signals are very low. The fragment ion at *m/z* 299 has been proposed to arise from the formation of an epoxide ring (13).

Figure 5B shows the negative-ion LSIMS MS/MS of the SQMG containing *cis* 13-hexadecenoic acid. Starting from *m/z* 553, a series of fragment ions is also clearly visible. The loss of 26 amu rather than 28 for two methylene groups, and a perturbation in the fragment-ion intensity pattern, indicates the site of a double bond. In general, the peak pattern for the acyl chain fragmentation is analogous to that following EI ionization of the corresponding nicotinate (Fig. 4). The formal cleavage of the double bond yields somewhat ambiguous signals between *m/z* 509–511, but the following peak at *m/z* 497 shows a difference of 26 mass units to the peak at *m/z* 523 and indicates that the double bond is located at the n-3 position. Note that the cleavage adjacent to the double bond

(vinylic cleavage) results in a decreased ion intensity for the fragment ion at *m/z* 497 (Fig. 5B) in a similar way to that observed in positive-ion EI mass spectrum for the nicotinate derivative. From *m/z* 497, a series of fragment ions corresponding to the formal elimination of successive methylene groups is visible down to *m/z* 371, a series analogous to that shown in Figure 5A, and indicating a straight chain saturated alkyl group.

This work indicates the difficulties in assigning the double bond position in n-3 fatty acyl moieties, by MS alone, in the absence of standards. The assignments can only be achieved by careful attention to fragment-ion mass-to-charge ratios, and relative signal intensities within a series of remote-site radical-induced or charge-remote fragmentations. Even then, the distinction between n-3 and n-4 monounsaturation is difficult, and must be confirmed by NMR analysis. Mass spectral analysis of SQDG n-3 monounsaturated fatty acyl groups has been demonstrated using two distinct methods of analysis, namely GC/MS analysis of nicotinates using EI ionization, and negative-ion LSIMS MS/MS for SQMG obtained from the corresponding SQDG by selective enzymatic cleavage. The latter procedure also greatly simplifies the MS/MS spectrum over that of the native SQDG. The analysis of SQMG by negative-ion LSIMS MS/MS is an alternative method for determination of carbon–carbon double bond positions in the *sn*-2 fatty acyl chain of SQDG.

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ANNOUNCEMENTS

CALENDAR

May 1996
XVIth Washington International Spring Symposium on Frontiers in Bioactive Lipids—'96, May 6–9, 1996, Washington, D.C., USA. Information: Dr. Jack Y. Vanderhoek, Chair—Symposium '96, Dept. of Biochemistry and Molecular Biology, The George Washington University, 2300 Eye St., N.W. RN 5530, Washington DC 20037-2337 USA (Phone: 202-994-2929; Fax: 202-994-8974; E-mail: jyvdh@gwis2.circ.gwu.edu.). Deadline for submission of abstracts: February 1, 1996.

July 1996
European Thrombosis Research Organisation and the International Society for the Study of Fatty Acids and Lipids in cooperation with Summer University of Maastricht, July 10–12, 1996, University of Maastricht, The Netherlands, presents "Lipids, Membranes and Thrombosis (fundamental basis of cardiovascular disease and its prevention)." Information: Gerard Hornstra, Dept. of Human Biology, P.O. Box 616, 6200MD Maastricht, The Netherlands (Phone: +31-43-88 16 25; Fax: +31-43-67 09 76; E-mail: G. Hornstra @ HB.RuLimburg.NL).

September 1996
7th International Congress on Phospholipids, Sept. 9–11, 1996, Brussels, Belgium. Program topic areas include Growth, Death and Cancer; Choline, Choline Phospholipids and the Brain; Importance of Phospholipids in Health and Disease. Information: AOCS Meetings Department, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

November 1996
AOCS Conference on PUFA in Infant Nutrition: Consensus and Controversies, Nov. 7–9, 1996, Barcelona, Spain. Information: Susan E. Carlson, scarlson@utmem1.utmem.edu; Robert A. Gibson, rgibson@paed_nnu.fmc.flinders.edu.au; Berthold Koletzko, Fax: 49-89-5160-4733; or AOCS Meetings Department, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

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REVIEW

n-3 Fatty Acids and Lipoproteins: Comparison of Results from Human and Animal Studies

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ABSTRACT: The impact of n-3 fatty acids (FA) on blood lipoprotein levels has been examined in many studies over the last 15 yr in both animals and humans. Studies in humans first demonstrated the potent triglyceride-lowering effect of n-3 FA, and these were followed up with animal studies to unravel the mechanism of action. This paper reviews the reported effects of n-3 FA on blood lipoproteins in 72 placebo-controlled human trials, at least 2 wk in length and providing 7 or less g of n-3 FA/day. Trials in normolipidemic subjects (triglycerides <2.0 mM; 177 mg/dL) were compared to those in hypertriglyceridemic patients (triglycerides ≥2.0 mM). In the healthy subjects, mean triglyceride levels decreased by 25% ($P < 0.0001$), and total cholesterol (C) levels increased by 2% ($P < 0.009$) due to the combined increases in low density lipoprotein (LDL)-C (4%, $P < 0.02$) and high density lipoprotein (HDL)-C (3%, $P < 0.008$). In the patients, triglyceride levels decreased by 28% ($P < 0.0001$), LDL-C rose by 7% ($P < 0.0001$), but neither total C nor HDL-C changed significantly. Although the effect on triglyceride levels is also observed in rats and swine, it is rarely seen in mice, rabbits, monkeys, dogs, and hamsters. Whereas n-3 FA have only a minor impact on lipoprotein C levels in humans, they often markedly lower both total C and HDL-C levels in animals, especially monkeys. These differences are not widely appreciated and must be taken into account when studying the effects of n-3 FA on lipoprotein metabolism.
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Fish oils rich in n-3 fatty acids (FA) have long been known to lower serum triglyceride levels in humans, but their effects on individual lipoprotein classes (low and high density lipoproteins, LDL and HDL, respectively) have been less clear. In 1989, a review appeared examining these questions based on the literature then available (1). In the ensuing years, more fish oil studies have been published which generally used lower intakes of n-3 FA for longer periods of time in larger groups of subjects. In the 1989 review (1), all studies were included regardless of whether or not they were placebo controlled and regardless of n-3 FA intakes or study durations. This present review incorporates the newer studies in a more critical analysis of only placebo-controlled trials using "practical" amounts of n-3 FA. This approach eliminated studies in which significant reductions in saturated FA accompanied the switch to the fish oil diet, potentially confounding the results (1). Also considered is the role of control oils (e.g., olive oil) as "placebos" vis-à-vis their impact on lipid and lipoprotein levels.

In addition to summarizing the most recent and well-designed human studies, it seemed appropriate to compare and contrast the results reported in humans with those found in experimental animals. It is the author's belief that significant confusion has crept into this field by the frequent (and often unconscious) extrapolation of animal data to the human situation. Animal experiments can obviously be useful in sorting out mechanisms of action, but when the effects seen in humans cannot be reproduced in the animal model, the significance of the animal data to human metabolism is unclear. If the effect elicited in humans does not occur in the animal model, it is inappropriate to use the latter to explore mechanisms underlying the former.

HUMAN STUDIES

The studies included in this summary were required to meet the following criteria: placebo-controlled with either crossover or parallel designs; no more than 7 g of eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) per day; study durations of at least two weeks; supplementation (not whole food substitution) studies; effects on triglyceride levels given; data from baseline as well as placebo and intervention periods reported; all types of subjects included (normals, hyperlipidemics, hypertensives, diabetics, and patients with coronary heart disease or renal disease); and English language articles. Although placebo-control was one of the criteria, two studies were included that were not placebo-controlled, Eritsland *et al.* (2) and Dehmer *et al.* (3). These were allowed because they included parallel, untreated control groups; they studied large numbers of patients (511 and 82, respectively) for relatively long periods of time (9 and 3 mon, respectively). Including these trials, there were 72 data sets taken

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Abbreviations: C, cholesterol; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; HDL, high density lipoprotein; HDL-C, HDL-cholesterol; LDL, low density lipoprotein; LDL-C, LDL-cholesterol; VLDL, very low density lipoprotein.

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from 60 separate publications (2–61). Approximately 70 studies with "before-and-after" designs were excluded.

In summarizing the lipid and lipoprotein data, the reported findings were weighted by study size in order to give the larger trials more influence. Briefly, the absolute change from baseline was calculated for placebo and fish oil treatments in each study. This change was multiplied by the number of subjects in the study to get an aggregate change for the whole study population. The aggregate lipid concentration at baseline (and after placebo) for all subjects was calculated by multiplying the number of subjects times the baseline (and placebo) lipid concentration (in mM). Then, these aggregates (both for lipid levels and for the changes) were summed across all studies. The overall, weighted, percentage changes were then calculated by dividing the aggregate change by the aggregate baseline (or placebo) value. This method was used for the crossover trials. For parallel group studies, the "baseline" lipid values were taken to be the average baseline for both treatment groups; otherwise the weighting procedure was the same as for the crossover trials.

Changes in lipid and lipoprotein levels from baseline to the end of the placebo, and the n-3 FA periods were compared by paired *t*-test. Dose-response relationships between n-3 FA intakes and fractional changes in triglyceride, and LDL-cholesterol (C) levels were determined by regression analysis. Both analyses were done using Microsoft Excel version 5.0.

Effects of n-3 FA vs. placebo on serum lipid and lipoprotein levels. The weighted percentage changes in lipids and lipoproteins according to patient type (normolipidemic vs. hypertriglyceridemic) across all studies are given in Figure 1. Mean intakes of n-3 FA were 3.7 g/d in the normals and 4.0 g/d in the patients. Triglyceride levels were reduced by 25–30% in both groups of subjects ($P < 0.0001$), and there was no significant difference between the change relative to

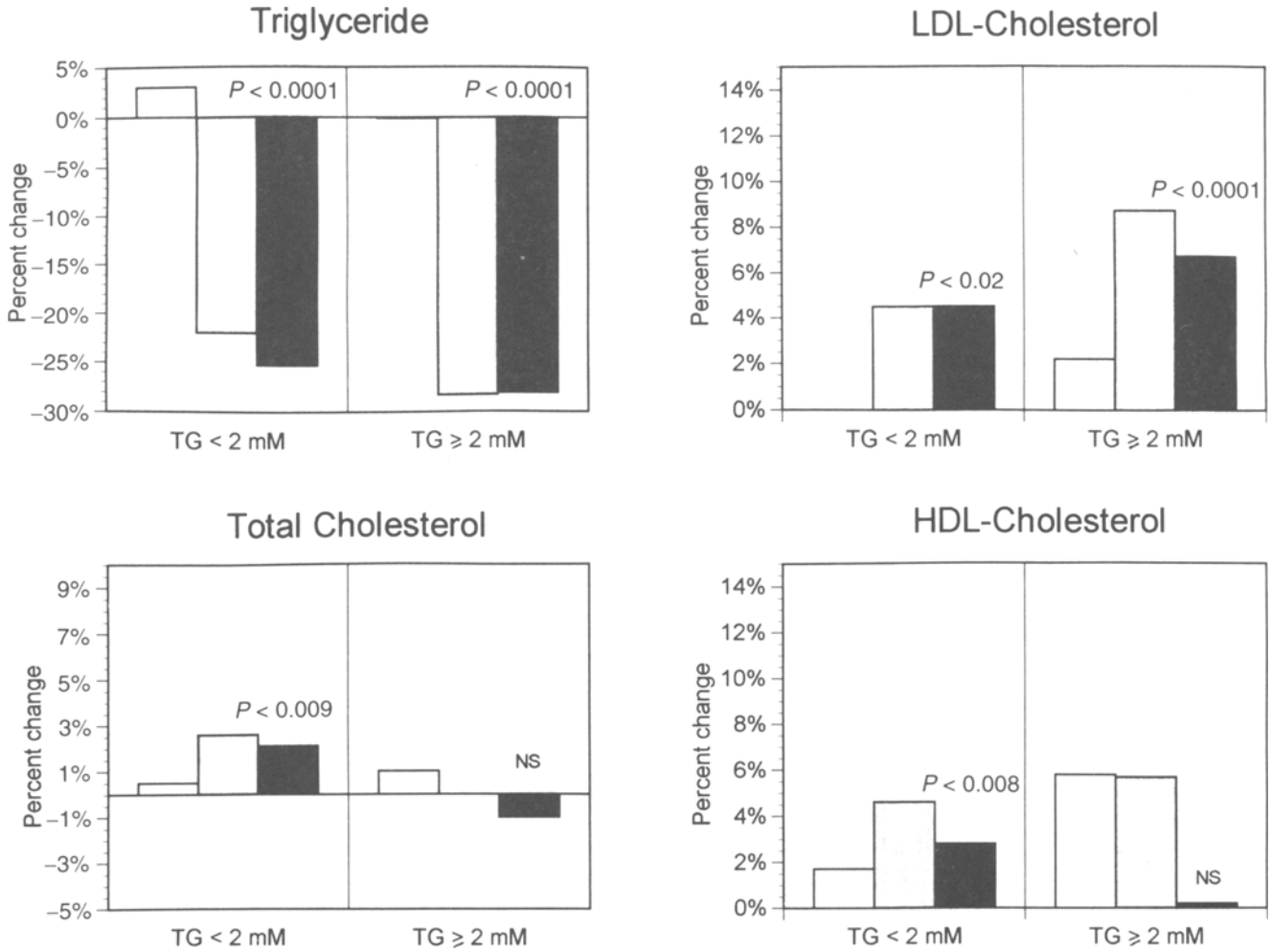


FIG. 1. The effects of n-3 fatty acids (FA) on serum lipids and lipoproteins in normotriglyceridemic (TG < 2 mM) and hypertriglyceridemic (TG ≥ 2 mM) subjects. [An average triglyceride (TG) molecular weight of 885 g/mol was used to calculate molarity, thus, 2 mM = 177 mg/dL]. Results from 72 placebo-controlled data sets in which over 1700 subjects were fed between 1 and 7 g of n-3 FA daily for at least two weeks are summarized. Percentage changes were calculated from weighted data allowing larger studies to contribute more to the mean effects. The three columns on each side of each graph show the change from baseline to placebo (open bars), the change from baseline to n-3 FA (gray bars), and the net difference between placebo and n-3 FA periods (black bars). *P* values are for the latter comparison; LDL, low density lipoprotein; HDL, high density lipoprotein.

baseline vs. that relative to placebo. Total C levels were slightly increased (about 2% vs. placebo, $P < 0.009$) in the studies with normolipidemic subjects, but were unaffected in the hyperlipidemics. Contributing to the rise in total C levels was the 4% rise in LDL-C in the normals ($P < 0.02$) and the 7% rise in patients ($P < 0.001$), respectively, and the 3% rise in HDL-C levels ($P < 0.008$) in the normals only; HDL-C levels were not affected in the patient studies. To find no rise in HDL-C in the face of an almost 30% reduction in very low density lipoprotein (VLDL) levels was unexpected in view of the relatively tight, inverse relationship between serum triglyceride and HDL-C levels (62). This finding suggests that n-3 FA may interrupt the metabolic connection between these lipoprotein fractions.

The inclusion of placebo groups was most helpful in deciphering the effects of n-3 FA on HDL-C levels in the patient studies because placebo "treatment" had the same effect as n-3 FA treatment, a 5–6% increase from baseline. Thus, the effect attributable to n-3 FA was nil. This finding calls into question the commonly held belief that polyunsaturated vegetable oils lower HDL-C levels (63); it appears that small amounts taken as supplements may do the opposite.

This exercise afforded the opportunity to ask if olive oil (the most commonly used placebo in these trials) was truly neutral vis-à-vis circulating lipid levels. In the 33 data sets that used olive oil (or its ethyl esters) (5,7,9,12,16–20,23, 25,27,31,33,34,36,37,40,42,44–47,49,51–55,57,59,61), the mean percentage changes (vs. baseline) in cholesterol, triglyceride, LDL-C, and HDL-C levels were –0.3, 2.3, –1.2, and 3.2%, respectively. Thus, about 10 g of olive oil per day had negligible effects on most lipoprotein fractions, with the possible exception of HDL-C. The similarity between these results for olive oil and those for all placebo oils combined (Fig. 1) suggests that polyunsaturated vegetable oils in general can be used as placebos in studies such as these. Furthermore, the relative neutrality of the placebos suggests that the reported changes in serum lipid levels in the over 70 "before-and-after" studies rejected from this analysis probably do reflect the true effects of n-3 FA (except possibly for HDL-C). Finally, the neutrality of the placebos supports the inclusion here of the Eritsland and Dehmer studies (2,3), which included parallel untreated control groups.

Although mean triglyceride levels decreased in 68 of the 72 data sets, the decreases were not always statistically significant. Triglycerides were reduced significantly in 73% of the studies in hypertriglyceridemic patients (2,3,15–18,20, 22–26,28–30,47,49–52,55,56,58), and in about 50% of studies with healthy subjects (4,5,7,8,10,11,13,32,36,39,43–45, 59,61). Thus, it was not uncommon for the decrease in blood triglyceride levels to not reach statistical significance, especially in studies in normolipidemic subjects. It should be noted, however, that often studies are not specifically sized to detect changes in triglyceride levels, especially studies designed to examine the effects of n-3 FA on nonlipid endpoints. In addition, smaller intakes of n-3 FA produce less triglyceride lowering (see below). Significant LDL-C increases were reported in 18% of the studies in normals (6,7,35,43,64) and 32% of the studies in patients (3,15–18, 22–24,29,52,55). LDL-C levels never decreased significantly vs. placebo in any trial. HDL-C rose significantly in 3% of studies (35,65) and never declined.

An important point from both therapeutic and regulatory standpoints is whether the triglyceride-lowering effect of n-3 FA is dose-related (66). Figure 2 shows that there is a statistically significant, inverse relationship between n-3 FA intakes and percentage triglyceride lowering both in volunteers ($r = 0.35$, $P < 0.001$) and in patients ($r = 0.50$, $P < 0.001$). Therefore, supplementary EPA + DHA (at least up to 7 g/d) lowers triglyceride levels in a dose-dependent manner. It is clear from these figures, however, that significant differences in

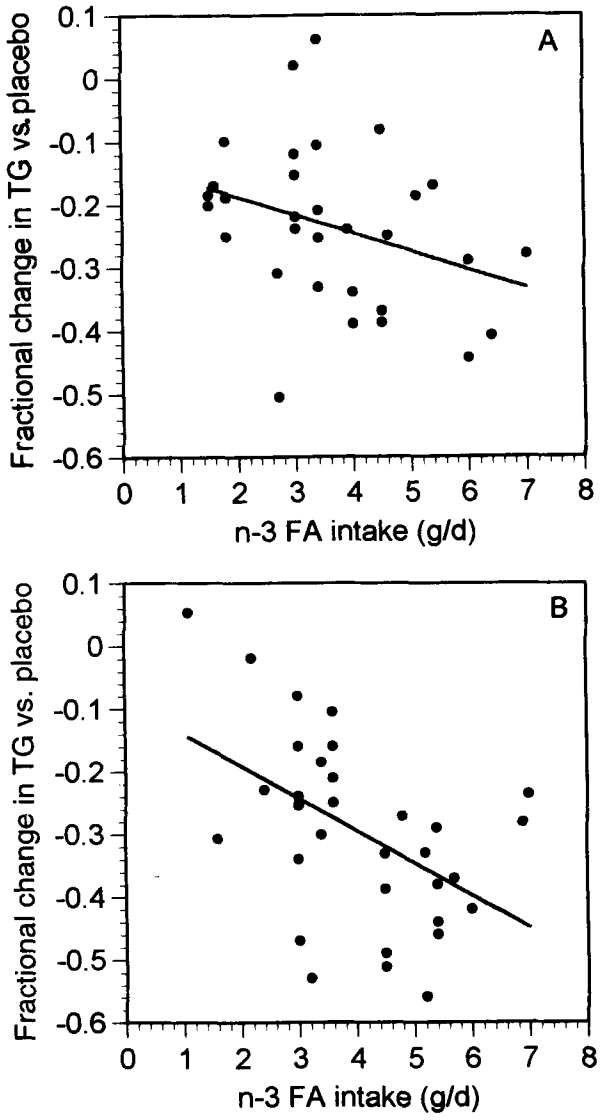


FIG. 2. Fractional changes (vs. placebo) in serum triglyceride levels as a function of n-3 FA intakes from studies in healthy subjects (A) and hypertriglyceridemic patients (B). Significant relationships were seen in both normals ($r = 0.35$, $P < 0.001$) and patients ($r = 0.5$, $P < 0.0001$). Abbreviations as in Figure 1.

response to the same dose were frequently observed (e.g., between 3 and 4 g/d in patient responses ranged from −5 to −52%).

There have been concerns that the hypotriglyceridemic effect may diminish with time (67). Figure 3 illustrates the percentage change in triglyceride levels over time for all studies combined. In order to "visually weight" each study, the points are drawn as a "bubbles," with the area of the bubble being proportional to the number of subjects studied. In this manner, the reader can immediately see not only the spread of the data but also the size of the studies from which results were obtained. From Figure 3 it is obvious that most studies have lasted 10 wk or less. The two largest studies (2,50) lasted 26 and 36 wk and provided 6.8 and 3.4 g of n-3 FA daily, respectively. Their results strongly suggest that the effect persists as long as supplementation continues. In the longest controlled trial, Sacks *et al.* (59) treated normolipidemic, coronary artery disease patients with n-3 FA or placebo for 112 wk. The n-3 FA group (n = 31) received 4.8 g of EPA + DHA daily, whereas the control group (n = 28) received 12 g of olive oil as a placebo. The fish oil produced small and nonsignificant increases in total, LDL-C, and HDL-C levels, and triglyceride levels were lowered by 30%. In an uncontrolled study providing about 3.3 g n-3 FA per day, Saynor and Gillott (68) reported that triglyceride reductions were maintained even after seven years of therapy. There can be little doubt that with continued supplementation, the hypotriglyceridemic effect persists.

Unlike the hypotriglyceridemic effect, the LDL-C raising effect does not appear to be dose-dependent. Figure 4 illustrates for patient studies only (where LDL-C most often increased) that there was no relationship between n-3 FA intake and percentage change in LDL-C levels ($r = 0.13$, nonsignificant). There was even a suggestion from the study of Leaf

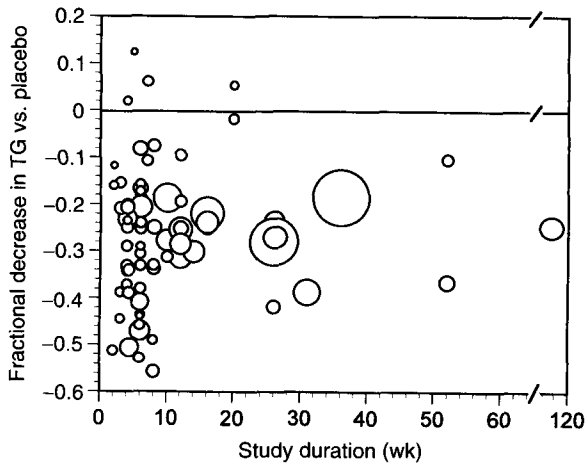


FIG. 3. A comparison of study duration to fractional triglyceride (TG) lowering in all data sets. Each set is represented by a "bubble," the area of which is proportional to the number of subjects in that data set. The largest "bubble" represents a study of 511 patients (Ref. 2). The 20–30% reduction in triglyceride levels persisted even in the longest study (28 mon) reported to date (Ref. 59).

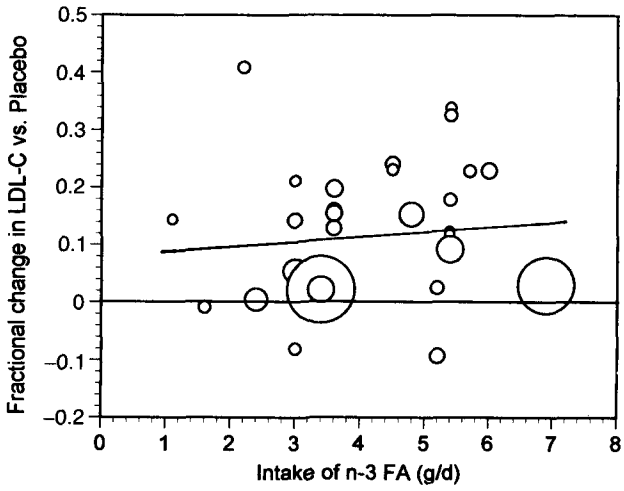


FIG. 4. Relationship between n-3 FA intake and fractional change in LDL-C in studies with hypertriglyceridemic patients. No relationship to dose is evident ($r = 0.13$, NS), with the largest studies showing no more than a 5% increase, even with 6.8 g of n-3 FA/d. See Figure 3 legend for a description of the data presentation, and Figure 1 for abbreviations; C, cholesterol.

et al. (50) that at LDL-C is not increased after six months of a large dose (6.8 g/d). Is this because the effect on LDL-C resolves with time? In the studies with normals (Fig. 5A), those lasting less than 10 wk showed a more consistent LDL-C response than those lasting more than 10 wk. In the study by Sacks *et al.* (59), there was no significant effect on LDL-C after 28 mon of 4.8 g of n-3 FA daily. In the patient studies (Fig. 5B), there was a fairly consistent rise in LDL-C levels of up to 30% in the shorter-term studies (<10 wk), but in most of the 6–12-mon studies (2,50,53), only a small and statistically insignificant 5% increase in LDL-C was noted. Germane to this question is the recent study by Morgan *et al.* (69), in which 7.5 g/d of n-3 FA increased LDL-C (vs. corn oil) at 6 wk, but not at 12 wk in 20 noninsulin-dependent diabetes mellitus patients. These findings suggest the LDL-C raising effect of n-3 FA supplementation may not persist as long as the hypotriglyceridemic effect.

One other interesting observation may be made from these studies in regard to effects of oil supplementation on body weight. Sacks *et al.* (59) gave their subjects an additional 12 g of oil daily for 28 mon, providing an extra 90,700 kcalories. This caloric load should have increased body weights by 11.8 kg, but only a 2 kg increase was noted. Smaller doses given for six (50) and nine (2) months should have increased body weights by 2 and 1.3 kg, respectively, but no change was found. It appears that either unconscious reductions in energy intake or increases in energy utilization accompanied oil (placebo or fish oil) supplementation.

In summary, using data derived only from placebo-controlled trials in which subjects were given less than 7 g of n-3 FA daily, the hypotriglyceridemic effect was substantial, it persisted over time, and was dose-dependent. LDL-C levels increased significantly in the short term, but appeared to

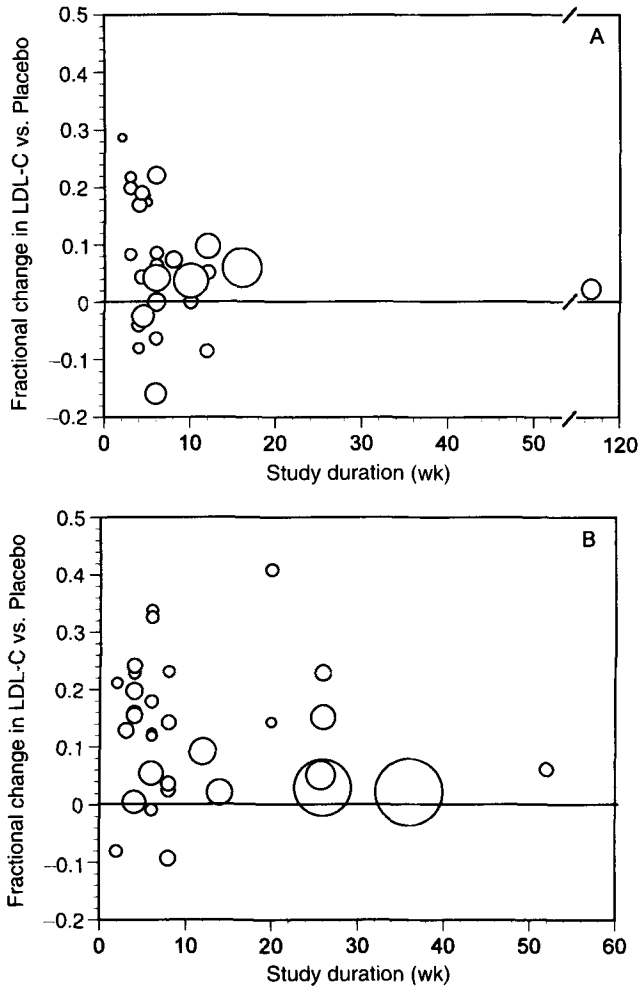


FIG. 5. Effect of study duration on LDL-C levels. For both normals (A) and patients (B), the net fractional change (vs. placebo) in LDL-C is given. Most of the larger studies reported minimal changes in LDL-C levels, especially those of longer duration. See Figure 3 legend for a description of the data presentation, and Figures 1 and 4 for abbreviations.

return toward baseline with time. Although there was no unique effect of n-3 FA on HDL-C levels, supplementation with any polyunsaturated oil would be expected to produce small increases compared to no supplementation.

ANIMAL STUDIES

Clearly there is a substantial body of consistent human data describing the effects of n-3 FA on serum lipid and lipoprotein levels. There is, likewise, a large body of animal data addressing this question, but as will be briefly described below, it is far less consistent, not only among, but also within, genera. Animal experimentation is frequently used to answer questions of ultimate relevance to humans. If the intervention used in an animal model does not produce the same effect as it does in humans, then the value of the model must be questioned. (It should be recalled here that the model is not only the species utilized, but also the experimental conditions employed, e.g., intake of n-3 FA, other dietary components, species-specific nutrient needs, duration of supplementation, period of fasting, etc.) Explorations of lipid-lowering mechanisms in such models may teach us little about how n-3 FA affect lipid metabolism in humans.

Triglycerides. Among laboratory animal species, only rats (70–72) and swine (73–75) generally responded to n-3 FA feeding with triglyceride lowering. Mice (76), rabbits (77–79), monkeys (80–82), and hamsters (83,84) usually did not exhibit a hypotriglyceridemic effect, and in dogs n-3 FA did not change any serum lipid parameter whatever (85–87). Interestingly, in African green monkeys, triglyceride levels rose during the n-3 FA feeding period compared to a lard diet (82,88), but did not change when compared to safflower oil (89).

LDL. LDL-C has not often been measured in animal studies, partly because in several commonly-used species (rats, mice, rabbits, dogs, hamsters), LDL is not the major C transporting lipoprotein; in these species, HDL seems to serve this function. When LDL-C was measured in swine fed fish oil, it usually was unaffected (90,91) or rose (73,92). In rabbits, LDL-C levels have been reported to go in all directions in response to fish oil feeding (93–95), and the most common effect noted in monkeys has been a significant LDL-C lowering (80,81,88), a response never noted in placebo-controlled humans.

HDL. Swine (73,96), monkeys (80–82,88,97,98), hamsters (83,84), and rats (99–101) most often responded to n-3 FA feeding with a substantial reduction in HDL-C whereas levels were generally unchanged in other species. Such a reduction stands in marked contrast with the neutral to positive effect on HDL-C levels noted in humans.

The reasons for this incomparability between animal and human responses to n-3 FA feeding are likely to be due to several factors. First, there are marked differences in lipoprotein metabolism among species (102), with (Westernized) humans being relatively unique with respect to their high ratio of LDL-C to HDL-C. Differences in susceptibility to oxidative stress (103), coupled with the failure of some investigators to provide adequate antioxidant protection (104), may have also played a role. In addition, the C contained in many n-3 FA preparations was often not balanced in the control diet, and this has been shown to have a significant impact on the n-3 FA response (84). Finally, the frequent use of very high intakes of n-3 FA in animal studies probably also contributed to the differences in response. A survey of animal experiments reporting the effects of fish oils on a wide variety of conditions (not just serum lipids) well illustrates this point (Fig. 6). When intakes were adjusted according to body surface area (105) and compared to a reasonable intake of 3 g/d in humans, then the prevalence of potential "overdosing" becomes evident. The effects in animals of "human equivalent" intakes of n-3 FA on lipid (or any other area of) metabolism have not been systematically investigated. Until they are, it will be difficult to produce pertinent animal models and to conduct meaningful mechanistic investigations to determine how n-3 FA impact human lipid metabolism.

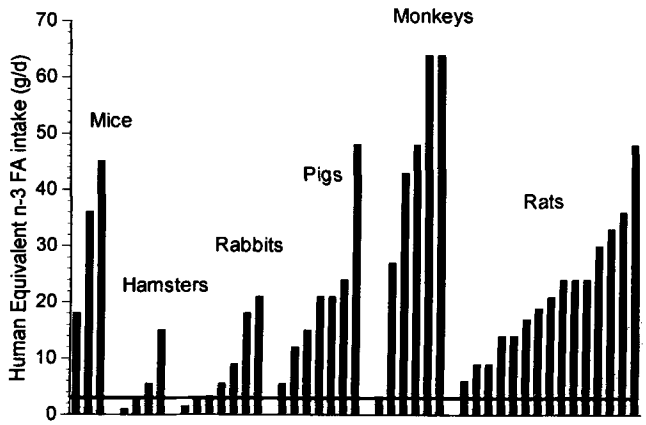


FIG. 6. Comparison of "human equivalent intakes" of n-3 fatty acids (FA) used in animal trials to the target intake of 3 g/d in humans (solid horizontal line). Reported intakes in animal studies were corrected for differences in body surface area according to Freiderich *et al.* (Ref. 105) in order to estimate the human equivalent intake.

Atherosclerosis. Although the focus of this review has been on fish oils and serum lipoprotein levels, the goal of many of the animal studies reviewed here was to determine the effects of n-3 fatty acids on arteriopathy [See Sassen *et al.* (106) for review]. Atherosclerosis (or surrogate phenomena such as intimal proliferation or monocyte adhesion to endothelial cells) has usually been inhibited by n-3 FA in swine (73,75,107,108), monkeys (81,82,88), and dogs (85–87,109). (Monkeys and swine are often considered to be the best models for human atherosclerosis.) In rabbits, neutral (77,79,93, 110,111), protective (94,95,112–115), and adverse (116–118) effects have all been reported. In the only mouse study reported, an antiatherosclerotic effect of n-3 FA was found (76).

In humans, there is significant (119–122), although not universal (123), epidemiological support for an antiatherogenic effect of n-3 FA. But there is only one prospective intervention study (124) in which the ability of fish oils to reduce cardiac morbidity and mortality was tested. In that trial, 2,033 men who had survived one heart attack were randomly assigned either to be given advice to eat two meals of oily fish per week or not. After two years, the fish advice group had a statistically significant 29% reduction in overall mortality. Both this study and the positive trials in swine and monkeys should compel us to mount a large-scale, secondary prevention trial with n-3 FA in humans.

It is important to note that in the animal studies, there was often a great discrepancy between the impact of n-3 FA on the extent of atherosclerosis and on blood lipoprotein levels. Less arterial disease in the presence of increased LDL-C and/or decreased HDL-C levels was commonly reported. The studies of Weiner *et al.* (73) in swine and Davis *et al.* (81) in rhesus monkeys are illustrative. In the former study, pigs were fed an atherogenic diet with or without 30 mL of cod liver oil for eight months. Thus, saturated fat intake was not reduced to make room for the fish oil; the latter was simply added to the diet (a vegetable oil control group would have been most helpful here as would a source of n-3 FA not rich in fat-soluble vitamins). Nevertheless, in this study, coronary arteriopathy was markedly reduced by fish oil feeding, and mean LDL-C levels increased from 300 mg/dL to nearly 400 mg/dL, and mean HDL levels decreased from 130 to 80 mg/dL, changes that would hardly be considered antiatherogenic. In the monkeys (81), the addition of fish oil (or the removal of coconut oil?) lowered HDL-C levels from 49 to 20 mg/dL, and yet the extent of disease was significantly reduced. In addition to having antiplatelet effects (125,126), n-3 FA treatment has also been reported to reduce macrophage uptake of monkey VLDL particles (127), reduce adhesion of leukocytes to hamster endothelium *in vivo* (128), and reduce the expression of adhesion molecules in vascular endothelial cells (129). Thus, these FA appear to have significant antiatherogenic potential independent of (despite?) their effects on lipid risk factors. It would be unfortunate indeed if future research with fish oils was hampered by paying more attention to their effects on surrogate risk factors than on atherosclerosis itself.

Conclusion. In humans, n-3 FA exert a significant hypotriglyceridemic effect which is dose-dependent and persistent. Effects on other lipoprotein fractions are relatively minimal and possibly transient. No animal model has yet been developed which mimics these effects. Accordingly, extreme care must be taken in order to avoid inappropriate extrapolations of animal findings to humans.

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ARTICLE

Study of the Δ12-Desaturase System of *Lipomyces starkeyi*

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ABSTRACT: The specific activity of the microsomal Δ12-desaturase system, which transforms oleic acid into linoleic acid, was about 16 pmol/min/mg protein. However, most of the total activity was nonsedimentable even after a 200000 × *g* centrifugation for 100 min. The study of various physicochemical parameters showed that this enzymatic complex, functioning optimally between pH 7 and 8, had low thermal stability. Ca^{2+}, which may cause an aggregation of the microsomes, and Hg^{2+} completely inhibited the activity, whereas Mg^{2+}, Mn^{2+}, and Zn^{2+} were activators. The Δ12-desaturase system was relatively specific toward oleic acid, though isomers of this fatty acid also had an action, either as substrates or as competitive inhibitors, on the activity of the system. The study of the effect of the exogenous oleoyl-CoA and elaidoyl-CoA on the specific activity of the Δ12-desaturase system showed a preference toward oleoyl-CoA.

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We have previously shown (1) that the lipids from yeast species belonging to the related genera *Lipomyces* and *Waltomyces* contain notable amounts of essential long-chain fatty acids (linoleic and α-linolenic acids), which are precursors of very long-chain polyunsaturated fatty acids such as dihomo-γ-linolenic and arachidonic acids found as traces. In this paper, we have also shown that oleic acid was the most abundant fatty acid in the lipids (40% of total fatty acids) of *Lipomyces* and *Waltomyces* and that its desaturation produced a relatively important amount of linoleic acid (19% of total fatty acids).

Although desaturases are key enzymes in the regulation of unsaturated fatty acid biosynthesis, studies dealing with the desaturation enzymatic complexes of microorganisms are not numerous, and most of the literature concerns the desaturases contained in rat liver microsomes. Among the microbial desaturase systems studied are those of *Bacillus* species (2), *Mortierella alpina* (3,4), *Mucor* sp. INMI (5), *Saccharomyces cerevisiae* (6), *Yarrowia lipolytica* (7,8), and *Cryptococcus curvatus* (9).

In the present work, we have studied the influence of various physical and chemical parameters on the specific activity of the microsomal Δ12-desaturase system of *Lipomyces starkeyi* CBS 1807 responsible for the formation of linoleic acid from oleic acid. The activity of the Δ6 desaturase system, which may desaturate oleic acid into $C_{18:2n-9}$, has not been studied here because this latter fatty acid was not detected in the fatty acid composition of the strain *L. starkeyi* CBS 1807 (1) nor in that of the microsomes prepared from the cells of this yeast.

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MATERIALS AND METHODS

Microorganisms. Lipomyces starkeyi CBS 1807 was obtained from the Centraal Bureau voor Schimmelcultures (Delft, The Netherlands).

Biochemicals. [1-^{14}C] Oleic acid (58 mCi/mmol) was obtained from Amersham (Amersham France, Les Ulis, France). It was diluted with cold oleic acid to a 10 mCi/mmol specific activity. Coenzyme A and ATP were purchased from Sigma (Sigma Chimie, Saint-Quentin Fallavier, France), and NADH was provided by Boehringer (Boehringer Mannheim, Meylan, France).

Culture medium and growth conditions. The strain was grown in medium G as described by Lomascolo *et al.* (1). Cultivation was conducted at 28°C for 15 h. At the end of the exponential growth phase, cells were harvested by centrifugation (7000 × *g* for 10 min) at 4°C and washed twice with a cold pH 7.5 buffered solution (buffer A) containing 0.05 M potassium phosphate, 0.65 M sorbitol, 1 mM EDTA, 1 mM DTT, and 1 mM $MgCl_2$.

Preparation of microsomes. The washed cells were suspended in 25 mL of buffer A and ground for 3 min in a CO_2 cooled Braun MSK bead grinder (Braun, Melsungen, Germany). The homogenate was then centrifuged at 25000 × *g* for 40 min, and the resulting supernatant fluid was centrifuged at 105000 × *g* for 100 min. The pellet, consisting in the microsomal fraction, was finally suspended in 2 mL of buffer A. This corresponded to 4.0 to 6.5 mg of protein per mL determined according to the procedure of Lowry *et al.* (10). Microsomes were used immediately for assays of desaturase activity. Aliquots of microsomes were kept at −20°C for 24 h before the determination of NADH:cytochrome *c* reductase (EC 1.6.2.1), succinate:cytochrome *c* reductase (EC 1.3.99.1), and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activities.

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Fatty acid extraction and analysis. Fatty acid methyl esters were obtained as follows. The assay reaction mixture (1.1 mL), added with 0.7 mL of an ethanolic solution of 4 M KOH, was saponified at 80°C for 1 h in sealed ampules. For the analysis of the microsomal fatty acid composition, 1.1 mL of a suspension of microsomes was used instead of the reaction medium. After removal of unsaponified substances with hexane, the mixture was acidified with 0.8 mL of 4 M HCl, and free fatty acids were extracted three times with 2.5 mL of hexane. After evaporation of the solvent using a flow of N_2, the residue was methylated in the presence of 0.75 mL of 12.5% boron trifluoride in methanol for 2 min at 90°C in sealed ampules. After cooling in ice, 0.75 mL of distilled water was added to decompose the excess of BF_3, and the fatty acid methyl esters were extracted three times with 1.5 mL of hexane. The solvent was then evaporated, and the samples were kept in 0.1 mL of hexane at −20°C under N_2. Fatty acid methyl esters were analyzed by gas chromatography as described by Lomascolo *et al.* (1).

Assays of NADH:cytochrome c *reductase, succinate:cytochrome* c *reductase, and glucose-6-phosphate dehydrogenase activities.* All assays were carried out at 30°C in a Uvikon 930 UV/Visible spectrophotometer (Kontron Instruments, Zürich, Switzerland). (i) NADH:cytochrome *c* reductase: the reaction mixture contained in a final volume of 1.5 mL: 50 mM Tris HCl buffer pH 7.2, 0.06 mM NADH, 0.06 mM ferricytochrome *c*, 3 mM NaN_3, and 0.05 mL of a suspension of microsomes. The reaction was started by the addition of microsomes, and the reduction of cytochrome *c* was followed by recording the absorbance increase at 550 nm. The millimolar extinction coefficient used for cytochrome *c* was 29 $L.mmol^{-1}.cm^{-1}$. (ii) Succinate:cytochrome *c* reductase: the reaction mixture for this enzyme assay was the same as above, except that NADH was replaced with disodium succinate. (iii) Glucose-6-phosphate dehydrogenase: the reaction mixture contained in a final volume of 1.5 mL: 100 mM Tris HCl buffer pH 7.5, 1 mM glucose-6-phosphate, 0.15 mM NADP, 20 mM $MgCl_2$, and 0.1 mL of microsomes. The reaction was started by the addition of microsomes, and the reduction of NADP was followed by recording the absorbance increase at 340 nm. The millimolar extinction coefficient used for NADP was 6.22 $L.mmol^{-1}.cm^{-1}$.

Assay of Δ12-desaturase activity. The reaction mixture (final volume, 1.1 mL) contained: 0.1 M potassium phosphate buffer, pH 7.2, 0.65 M sorbitol, 1 mM DTT, 5.5 mM $MgCl_2$, 6.5 mM ATP, 0.5 mM CoA, 0.7 mM NADH, 18–20 μM [1-^{14}C] oleic acid (in 5 μL of ethanol, i.e., 0.2 μCi), and 0.15 mL of microsomes (0.6–1 mg protein).

The reaction was started by the addition of [1-^{14}C] oleic acid and was performed for 10 min at 40°C (except for the study of the effects of temperature on activity) with constant shaking in air. The reaction was stopped by adding 0.7 mL of 4 M KOH in ethanol. For the study of the influence of cations, the assay reaction medium (potassium phosphate buffer pH 7.2, sorbitol, DTT, $MgCl_2$, ATP, CoA and NADH, microsomes and cation) was preincubated for 10 min before adding [1-^{14}C]. The preincubation time was 2 min for the study of the influence of chemical amino reactive reagents. After saponification and methylation of fatty acids as described above, fatty acid methyl esters were separated by argentation thin-layer chromatography using toluene/acetonitrile (97:3, vol/vol) as mobile phase. Plates of Silica gel 60G (Merck, Darmstadt, Germany) were impregnated with 10% silver nitrate according to Wilson and Sargent (11). Commercial standards of $C_{18:0}$, $C_{18:1}$, and $C_{18:2}$ were cochromatographed on each plate. Following their visualization using 2′-7′-dichlorofluoroscein, the spots corresponding to saturated, monounsaturated, and biunsaturated fractions were separately scraped off the plates. The silica gel containing the fractions was suspended in Packard Instagel Plus scintillation liquid (Packard, Rungis, France) added with 77% (by vol) of distilled water in vials protected from light in order to prevent the formation of silver oxide. Radioactivity was counted after one hour in a 1600 TR Liquid Scintillation Analyzer (Packard Instruments S.A., Rungis, France). From the amount of radioactivity found in the diene fraction vs. the radioactivity recovered from the monoene fraction, the enzyme activity could be determined and expressed as picomoles of oleic acid converted to linoleic acid per min and per mg of microsomal proteins.

For each activity value given in this paper, at least two microsomal preparations obtained from different cultures were used, with at least three independent measurements made on each preparation. The activity value given was the mean of these six or more determinations.

The diene fraction obtained by thin-layer chromatography after 0 and 10 min of reaction at 40°C (standard conditions) has been identified as linoleic acid ($C_{18:2n-6}$) by gas chromatography by comparison of the retention time of the resulting peak with that of a commercial sample of pure linoleic acid.

RESULTS AND DISCUSSION

Localization of Δ12-desaturase system activity. Little activity (<2%) was associated with the mitochondrial fraction (25000 × *g* pellet). Table 1 shows that only a part of the activity of the Δ12-desaturase system was contained in the microsomal 105000 × *g* pellet. Total Δ12-desaturase system activity was 30 times higher in the 25000 × *g* supernatant (which contained microsomes plus cytosol) and 16 times higher in the 105000 × *g* supernatant than in the 105000 × *g* pellet. The specific activity measured on microsomes obtained by a 200000 × *g* centrifugation was the same as that found in microsomes contained in the 105000 × *g* pellet (16 pmol/min/mg protein).

The measurement of NADH:cytochrome *c* reductase, succinate:cytochrome *c* reductase, and glucose-6-phosphate dehydrogenase activities verified the nature of the pellet obtained after centrifugation at 105000 × *g*. A rotenone-insensitive NADH:cytochrome *c* reductase is also a characteristic enzyme from the endoplasmic reticulum in rat liver (12), and of the microsomes of mammalian and plant cells in which

TABLE 1
Distribution of Δ12-Desaturase System Activity

Cell fraction	Protein content (mg)	Formation of linoleic acid: Total activity (nmol/min^{-1})	Formation of linoleic acid: Specific activity[a] (pmol/min/mg protein)
Supernatant at 25000 × *g*	40	4.8 (100%)	131.1 ± 11
Microsomes, pellet at 105000 × *g*	9	0.2 (4%)	20.8 ± 1
Supernatant at 105000 × *g*	37	2.9 (60%)	74.1 ± 14

[a]Values of specific activity are given as mean ± SEM (n = 3).

it is linked to cytochrome b_5 and is involved in the fatty acid desaturation (13). Desaturases in the microsomal electron transport system occur together with NADH:cytochrome b_5 reductase and cytochrome b_5. Our results show that the activity of NADH:cytochrome *c* reductase measured in 105000 × *g* pellet extracts which had been frozen at –20°C was the same with or without rotenone (7.5 µL of a 1 mg.mL^{-1} ethanolic solution): 0.021 µmol cytochrome *c* reduced/min/mg protein ±0.004 (n = 9). On the other hand, the succinate:cytochrome *c* reductase, characteristic of mitochondrial activities (14), did not have any activity in the microsomal pellet, whereas the specific activity of this enzyme was 9 nmol cytochrome *c* reduced/min/mg protein in the 25000 × *g* pellet (i.e., in the mitochondria). Finally, glucose-6-phosphate dehydrogenase, characteristic of the microsomes plus cytosol fraction (15) was present in the microsomal pellet and the supernatant of the 105000 × *g* centrifugation (0.1 ± 0.02, n = 4 and 0.37 ± 0.13, n = 4 µmol NADP/min/mg protein, respectively). The 105000 × *g* pellet was thus effectively composed of microsomes and free of mitochondria. Unless otherwise specified, this pellet was used in all the subsequent studies.

Fatty acid composition of microsomes. The long-chain fatty acid composition of microsomes is given in Table 2: oleic acid, linoleic, and palmitic acids represented more than 80% of total fatty acids. This composition was similar to that of the whole cells of *L. starkeyi* (1).

Δ12-Desaturase system specific activity in standard conditions. We have verified that the specific activity (16 pmol/min/mg protein ± 4, n = 10) was proportional to the reaction time when measured after up to 10 min in the standard reaction conditions described in the Materials and Methods section. This specific activity was the same when the microsomal protein amount in the reaction mixture was doubled.

The specific activity was in the same range as that found in *M. alpina* under similar conditions (3). On the other hand, it was threefold lower than that of *Mucor* sp. INMI (5) and sixfold lower than that of *Y. lipolytica* (7). Culture conditions, particularly aeration and temperature, may influence the production or the activity of the Δ12-desaturase system. The different procedures used for the obtention of microsomes may also result in differences of activity. Notably, as most of the Δ12-desaturase activity was found as a nonsedimentable form in the 105000 × *g* and 200000 × *g* supernatants, i.e., not in the standard microsomal fraction, the procedures for disruption may influence the activity found in the microsomes. A high proportion of Δ12-desaturase activity was also observed in 160000 × *g* and 200000 × *g* supernatants of microsomal preparations from *Y. lipolytica* (7).

Effects of temperature and pH on the specific activity of the Δ12-desaturase system: (i) Influence of temperature: the reaction was performed in the same conditions as described for the standard reaction but for different temperatures. The results given in Figure 1 show that the optimal temperature was 40°C for 10 min of reaction. The study of the thermal denaturation of the Δ12-desaturase system was realized by incubating the microsomal extracts for 15 and 30 min at various temperatures (30 to 45°C) before adding labelled oleic acid. After this treatment, the enzyme activity was determined in the standard conditions (Fig. 2A). Above 45°C, the Δ12-desaturase system did not show activity after 10 min. This sensitivity to temperatures higher than 40°C is likely to be due to the low apparent activation energy of the thermal denaturation of the enzyme complex (31 kJ.mol^{-1} ± 19, n = 3), determined using an Arrhenius plot (Fig. 2B). (ii) Influence of pH: the reaction was realized as described for the standard reaction but for pH values varying from 5 to 9. The buffers used were citric acid/disodium phosphate 0.1 M (pH 5), potassium phosphate 0.1 M (pH 6–8), and Tris HCl 0.1 M (pH 9). The enzymatic system functioned optimally when microsomes were placed in a reaction medium with a pH between 7 and 8 (Fig. 3), corresponding to the usual intracellular pH range. It is to be noted that the pH at the active sites of the Δ12-desaturase system in the

TABLE 2
Long-Chain Fatty Acid Composition of Microsomes of *Lipomyces starkeyi* CBS 1807[a]

Fatty acid	Percentage of total fatty acids[b]
$C_{14:0}$	1.3 ± 0.21
$C_{15:0}$	0.2 ± 0.02
$C_{16:0}$	27.0 ± 2.5
$C_{16:1}$ (*c*9)	2.3 ± 0.35
$C_{17:0}$	0.2 ± 0.03
$C_{17:1}$ (*c*10)	0.2 ± 0.03
$C_{18:0}$	5.0 ± 0.39
$C_{18:1}$ (*c*9)	35.0 ± 0.74
$C_{18:2}$ (*c*9, *c*12)	22.1 ± 0.34
$\alpha C_{18:3}$ (*c*9, *c*12, *c*15)	1.4 ± 0.25

[a]Centraal Bureau voor Schimmelcultures (Delft, The Netherlands).
[b]Values are given as mean ± SEM (n = 3).

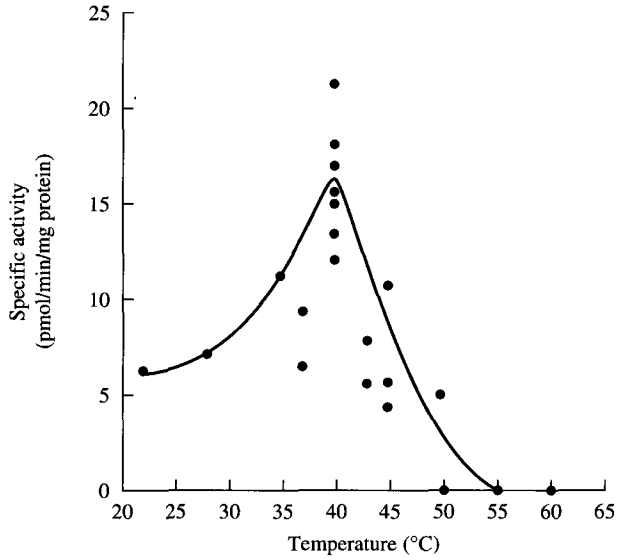


FIG. 1. Influence of temperature on the specific activity of the Δ12-desaturase system.

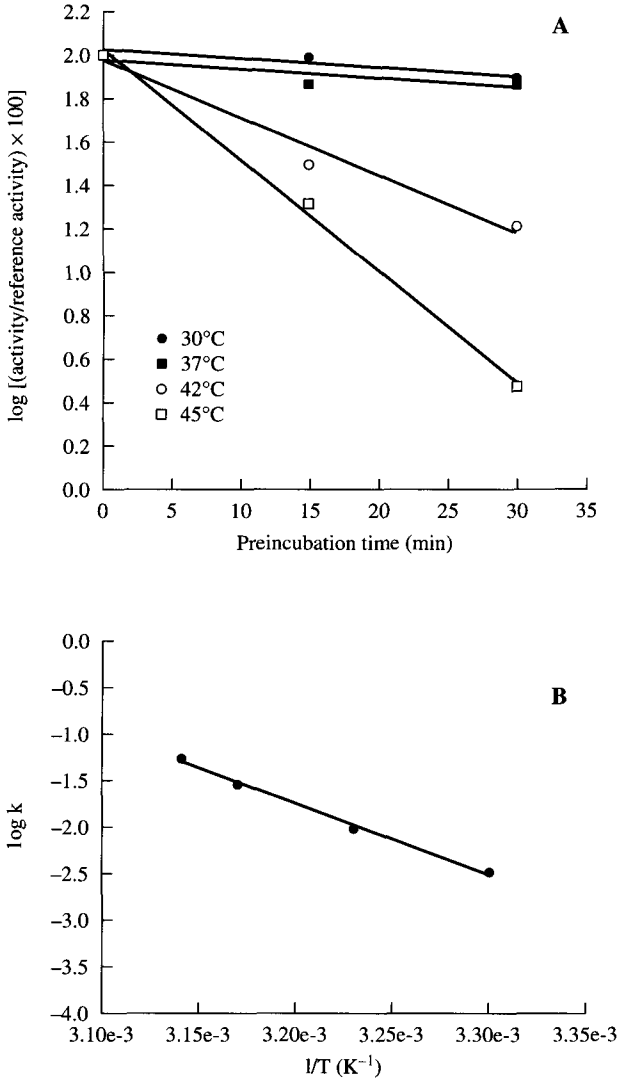


FIG. 2. (A) Thermal denaturation. Residual specific activity of the Δ12-desaturase system after 15 and 30 min of preincubation at different temperatures (30, 37, 42, and 45°C). The reference activity was measured in the standard reaction conditions (10 min at 40°C). (B) Thermal denaturation of the Δ12-desaturase system (Arrhenius plot).

microsomes may be different from the pH of the reaction medium.

Apparent K_m. By varying the concentration of labelled oleic acid in the reaction mixture, an apparent K_m value of 50 μM (±9, n = 3) was determined from the Lineweaver-Burk plot represented in Figure 4. It was in the same range as that found for *Y. lipolytica* (7).

Influence of effectors. Table 3 indicates the relative Δ12-desaturase specific activity (%) in the presence of different cations in the reaction mixture, compared to a reference realized in standard conditions. The activity was completely inhibited by Hg^{2+}. Among the other cations, Ca^{2+} presented the most important effect since it inhibited 80% of the activity. However, it is to be noted that this cation was employed in the protocol of Käppeli *et al.* (16) in order to precipitate the microsomal fraction, recovered by a rapid centrifugation at 15000 × *g*. It may thus mean that in the presence of Ca^{2+} in the reaction mixture, microsomes formed aggregates of lower specific activity. The fact that EDTA (Table 4), which is a metal chelating agent, inhibited 60% of activity indicated that a cationic ion is necessary to the functioning of the Δ12-desaturase system. Mg^{2+} is generally used in desaturase assays. In our case, the activity in the presence of 3 mM Mg^{2+} was 30% lower than in the standard conditions (5.5 mM). However, Zn^{2+}, and more especially Mn^{2+} had, at the concentration of 3 mM, a same or higher activating effect as 5.5 mM Mg^{2+}.

Table 4 gives the relative activity (%) of the Δ12-desaturase complex in the presence of effectors reacting with peculiar amino acid residues and of EDTA. An inhibitory effect was observed in the presence of *N*-ethylmaleimide (100%), iodine (90%), Woodward reagent K (i.e., *N*-ethyl-5-phenyl-

TABLE 3
Influence of Different Cations at 3 mM on the Activity of the Δ12-Desaturase System

Cations (chloride salts)	Relative Δ12-desaturase system activity[a] (%)
Mn^{2+}	123 ± 6
Zn^{2+}	106 ± 3
Hg^{2+}	0
Mg^{2+}	71 ± 2
Co^{2+}	59 ± 2
Cu^{2+}	97 ± 3
Ca^{2+}	19 ± 2

[a]This percentage was the ratio between the activity obtained in presence of each cation and the activity measured in the standard conditions (16 pmol/min/mg protein). Values are given as mean ± SEM (n = 3). Each cation was incubated 10 min with microsomes before adding oleic acid.

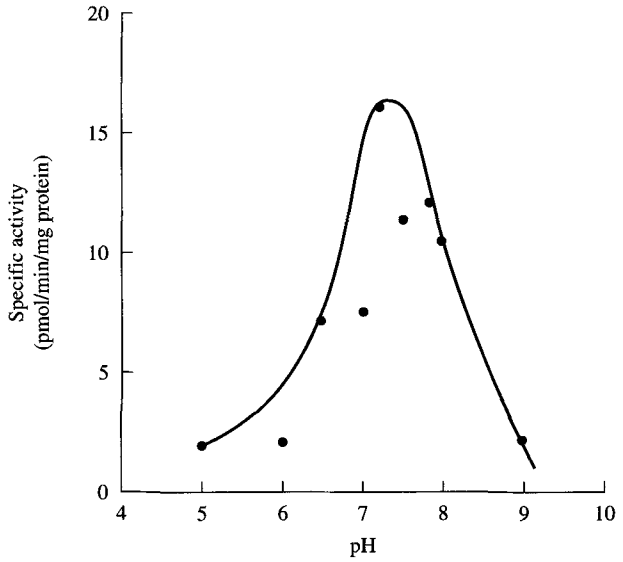


FIG. 3. Influence of pH on the specific activity of the Δ12-desaturase system.

isoxazolium 3′-sulfonate, 90%), *N*-bromosuccinimide, and iodoacetic acid (50–60%). Cystein (characterized by *N*-ethylmaleimide and iodoacetic acid), tyrosine (iodine and *N*-bromosuccinimide), aspartic and glutamic acids (the Woodward reagent K), and histidine (iodoacetic acid and *N*-bromosuccinimide) thus seem to be necessary to the reaction. It is to be noted that the inhibitory effect of iodine may also be due to a reaction with the double bonds of the unsaturated fatty acids involved in the reaction.

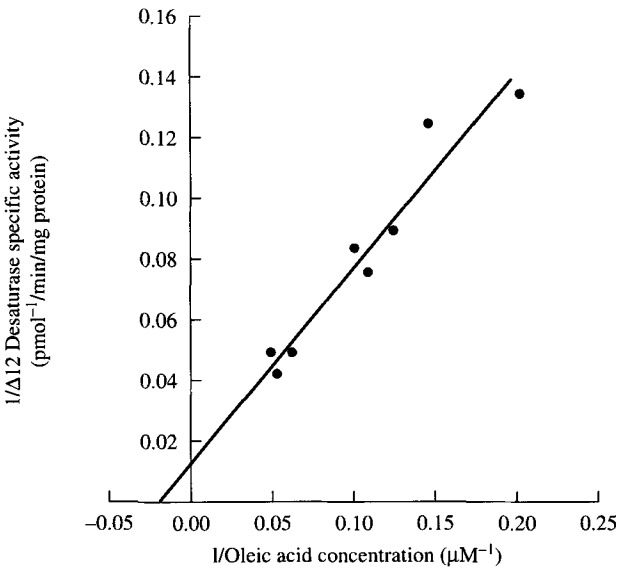


FIG. 4. Δ12-Desaturase system specific activity in standard reaction as a function of oleic acid concentration: double-reciprocal (Lineweaver-Burk) plot.

TABLE 4
Influence of Different Chemical Amino Reactive Reagents and of EDTA on the Activity of the Δ12-Desaturase System

Reagent (final concentration 3 mM)	Relative Δ12-desaturase system activity[a] (%)
2,4-Pentanedione	111 ± 3
N-bromosuccinimide	57 ± 5
Woodward reagent K[b]	13 ± 2
2,5-Dimethoxytetrahydrofurane	97 ± 4
Phenylmethylsulfonyl fluoride	86 ± 4
Iodoacetic acid	55 ± 4
N-ethylmaleimide	0
Trifluoroacetic anhydride	62 ± 4
2-Mercaptoethanol	75 ± 5
Iodine	10 ± 2
4-Dimethylaminobenzaldehyde	96 ± 4
EDTA	42 ± 1

[a]This percentage was the ratio between the activity obtained in presence of each reagent and the activity measured in the standard conditions (16 pmol/min/mg protein). Values are given as mean ± SEM (n = 3).
[b]Woodward reagent K: *N*-ethyl-5-phenylisoxazolium 3′-sulfonate. Each effector was incubated 2 min with microsomes before adding [^{14}C] oleic acid.

Effect of isomers of oleic acid and of oleoyl-CoA and elaidoyl-CoA. In our conditions of determination of the activity of the Δ12-desaturase system, at least two activities are involved: in a first step, acyl CoA synthetase catalyzes the formation of oleoyl-CoA from oleic acid and CoA, then oleoyl-CoA is desaturated into linoleoyl-CoA (we have verified that no desaturation occurred when CoA was absent in the reactant medium). In an attempt to evaluate the substrate specificity of the Δ12-desaturase system, the desaturation of oleic acid into linoleic acid was measured in the presence of equimolar quantities (0.02 mM) of labelled oleic acid and cold isomers of this fatty acid (Table 5). The Δ12-desaturase activity was about 25% inhibited in the presence of petroselinic, petroselaidic, and *cis*-vaccenic acids. Elaidic acid had a slightly higher inhibitory effect, whereas *trans*-vaccenic acid inhibited the reaction by only 10%. If the system had the same affinity toward these isomers as toward oleic acid, the activity would be 50% inhibited. There is thus at least one enzyme in the desaturase complex which presents a higher affinity toward oleic acid than its isomers. This enzyme may be the acyl-CoA synthetase, the Δ12 desaturase, or an intermediary enzyme, such as an oleoyl-CoA acyl transferase responsible for the formation of 1-acyl 2-oleoyl-*sn*-3-phospholipids as suggested by Ferrante *et al.* (7). It is to be noted that mammalian liver and *Bacillus* acyl-CoA synthetases are known to be very nonspecific (17).

In a second set of experiments, we have studied the influence of the addition of cold oleoyl-CoA or elaidoyl-CoA to the reaction mixture containing microsomes (105000 × *g* pellet) or the 105000 × *g* supernatant. Inhibition of the microsomal desaturase complex by 0.02 mM oleoyl-CoA and elaidoyl-CoA, which were supposed to compete with the [^{14}C] oleoyl-CoA produced by the acyl-CoA synthetase, was only 12 and 3%, respectively (Table 5). This was lower than expected, as cold oleoyl-CoA should have reduced the mea-

TABLE 5
Influence of the Addition of Different Isomers of Oleic Acid and of Oleoyl-CoA and Elaidoyl-CoA on the Activity of the Δ12-Desaturase System in the Presence of 0.02 mM [^{14}C] Oleic Acid

Fatty acids and acyl-CoA	Final concentration (mM)	Percentage of residual Δ12-desaturase system activity[a]
Cold oleic acid (*cis*-9)[b]	0.02	51 ± 4
Elaidic acid (*trans*-9)[b]	0.02	70 ± 4
Petroselenic acid (*cis*-6)[b]	0.02	78 ± 6
Petroselaidic acid (*trans*-6)[b]	0.02	76 ± 5
cis-Vaccenic acid (*cis*-11)[b]	0.02	77 ± 2
trans-Vaccenic acid (*trans*-11)[b]	0.02	88 ± 3
Oleoyl-CoA[b]	0.02	88 ± 1
Elaidoyl-CoA[b]	0.02	97 ± 1
Oleoyl-CoA[b]	0.20	0
Elaidoyl-CoA[b]	0.20	0
Oleoyl-CoA[c]	0.02	45 ± 4
Elaidoyl-CoA[c]	0.02	51 ± 2

[a]This percentage was the ratio between the activity obtained in presence of each isomer of oleic acid or of each molecule of acyl-CoA and the activity measured in the standard conditions. Values are given as mean ± SEM (n = 3).
[b]Reactions were realized with microsomes (105000 × *g* pellet).
[c]Reactions were realized with the 105000 × *g* supernatant.

sured activity by at least 50% by simple dilution of the [^{14}C] oleoyl-CoA. This indicates that the concentration of exogenous oleoyl-CoA at the active site of the desaturase complex was about ten times lower than that of the [^{14}C] oleoyl-CoA produced from the 0.02 mM [^{14}C] oleic acid. The access of exogenous acyl-CoA to the desaturase complex, probably located at the inner side of the microsomal membrane, was thus a limiting step, certainly due to the hydrophilic properties of acyl-CoA. When oleoyl-CoA and elaidoyl-CoA were added at a higher concentration (0.2 mM), a full inhibition of the measured activity was observed. In this case, the inhibition was much stronger than expected from the results obtained with 0.02 mM: if the concentration of exogenous acyl-CoA were 10-fold higher at the active site of the Δ12-desaturase, the measured activity should have been at least 50% of the reference activity. Two hypotheses could explain the 100% inhibition that was observed: (i) an inhibition of acyl-CoA synthetase by acyl-CoA, which would reduce the amount of radioactive acyl-CoA produced from [^{14}C] oleic acid; and (ii) the presence of a transport system for acyl-CoA functioning for acyl-CoA concentrations higher than 0.02 mM.

In the absence of integral microsomes, in the 105000 × *g* supernatant, the inhibition by 0.02 mM acyl-CoA was about 50%. This indicates that the access of acyl-CoA to the desaturation system is probably more direct in the supernatant fraction, which is consistent with the hypothesis that the supernatant contains finely dispersed fragments of endoplasmic reticulum (too small to form microsomes), or a solubilized form of the desaturation system: in both cases, the limiting step of the susbstrate penetration in or through the microsomal membrane is absent. This faster access to the enzymatic system also applies to oleic acid, which could partly explain the high desaturase activity of the supernatant compared to microsomes.

The influence of the concentration of exogenous oleoyl-CoA or elaidoyl-CoA on the activity of desaturation of [^{14}C] oleic acid is shown on Figure 5. The experimental data concerning oleoyl-CoA were in quite good agreement with the model according to which the exogenous oleoyl-CoA simply dilutes the [^{14}C] oleoyl-CoA produced from [^{14}C] oleic acid by the acyl-CoA synthetase. The data concerning elaidyl-CoA were also in agreement with the dilution model, but the activities were always higher than in the presence of oleoyl-

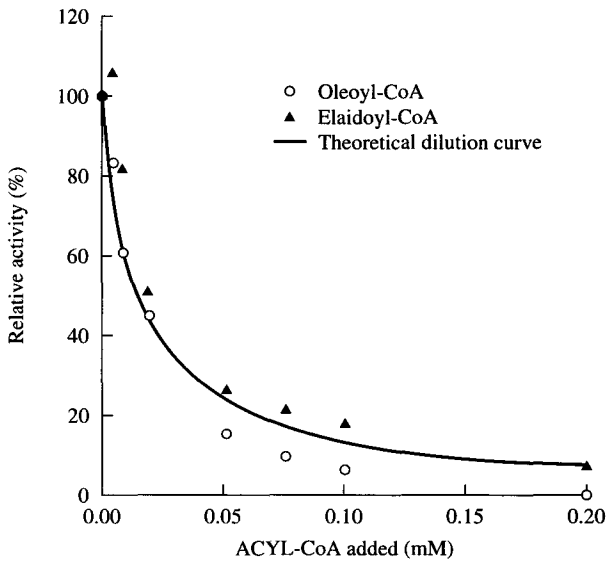


FIG. 5. Influence of the addition of cold oleoyl-CoA (○) or elaidoyl-CoA (▲) on the activity of desaturation of 0.02 mM [^{14}C] oleic acid. The dotted line represents the theoretical reduction of this activity by dilution of the intermediate [^{14}C] oleoyl-CoA with the cold oleoyl-CoA added.

CoA. This probably reflected a higher affinity of the Δ12 desaturase, or of an intermediary enzyme such as an oleoyl-CoA acyltransferase, for oleoyl-CoA than for its *trans* isomer. The study of the substrate specificity of the Δ12 desaturase system is difficult due to the involvement of numerous enzymes. However, the use of the 105000 × *g* supernatant seems to facilitate this study.

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Lipolysis and Heterogeneous Catalysis. A New Concept for Expressing the Substrate Concentration

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ABSTRACT: A new concept is proposed for quantifying the substrate concentration during heterogeneous catalysis of the kind which occurs during lipolysis. The number of molecules of protein (enzyme) adsorbable to the lipid substrate interface per unit of volume was evaluated and defined as a volumetric concentration of protein (enzyme) binding site (PEBS). Using porcine pancreatic lipase (EC 3.1.1.3) as a model enzyme, the maximal PEBS concentration was measured under various assay conditions by determining the saturation of the lipid substrate with the enzyme. Abacuses correlating the lipid substrate concentration (M) with the PEBS concentration (M) under each experimental conditions were used to express the kinetic data in terms of a volumetric concentration of PEBS. Comparisons could thus be made between data obtained with various enzymes and lipid interfaces because they were expressed with the same unit. In the case of pancreatic lipase, using triolein and tributyrylglycerol as substrates, K_m values of 2.7 and 7.5 nM PEBS were obtained, respectively, and K_D values ranging around 9 nM PEBS were also obtained from Scatchard plots. In addition, the average superficial density of PEBS was found to be 10×10^{11} molecules • cm^{-2}, which is a value commonly obtained with structural proteins and enzymes adsorbed to an acylglyceride–water interface, this finding supports the idea that the PEBS concept represents the room in which the protein molecule adsorbs at the lipidic interface.

Lipids 31, 261–267 (1996).

Lipolytic enzymes are characterized by their action at oil–water interfaces where their substrate is present. In situations of this kind, which are typical of heterogeneous catalysis, it is difficult to evaluate the kinetic parameters, particularly the affinity of the lipolytic enzymes for their lipidic substrates. The difficulties are due primarily to the fact that the lipidic substrate can generally be only poorly described in terms of quantitative and qualitative parameters.

Benzonana and Desnuelle (1) were the first to demonstrate that there exists a relationship between the "surface concentration" of the lipidic substrate and the lipolytic activity; in addition they pointed out that the intrefacial area in a volume unit was not the only significant variable in the process: this finding helped to focus attention on these other variables such as the nature of the lipidic surface. Monolayer studies at the air–water interface, in situations in which the area of the surface formed by the lipidic substrate is known with precision, have highlighted the variability of the protein (enzyme)–lipid interactions which can occur with the same amount of surface of the same lipid, depending on the lipid packing, for example (2–4). As the result of this variability, it is still difficult to compare kinetic data obtained under different experimental conditions, and no dissociation constant, K_D, or thermodynamic values have been published to date which might facilitate the making of comparisons between the interactions involving various enzymes and substrates and give better insight into the biological and physicochemical processes underlying these interactions.

The aim of the present study was to propose a convenient means to express the "substrate concentration" in heterogeneous catalysis which would allow, at the same time, to take into account the parameter "surface concentration" and the qualitative parameters of the protein (enzyme)–lipid interaction. Under each type of assay conditions, the maximal capacity of the lipid substrate to bind the enzyme molecules was evaluated from saturation curves, and the corresponding concentration of adsorbable molecules was taken to be the true substrate concentration. This transformation facilitates the treatment of the kinetic data and permits more satisfactory comparisons between (i) various Michaelis constants, K_m, measured on various lipidic substrates, and (ii) the K_D of various proteins at a given lipidic interface.

MATERIALS AND METHODS

Assay of lipase activity. Lipase activity was assayed potentiometrically using a pHstat apparatus (Radiometer, Copenhagen, Denmark). Assays were carried out at pH 8.0 and at 25°C with emulsified glycerol tributyrate (Fluka, Buchs, Switzerland) or preemulsified triolein (10%, vol/vol) in gum arabic (10%, wt/vol in water). The aqueous phase consisted

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Abbreviation: PEBS, protein (enzyme) binding site.

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of 2.5 mM Tris-HCl buffer pH 8.0 containing 5 mM $CaCl_2$ and 10 mM NaCl. The total volume of the assay medium, including the lipid, was 25 mL. The hydrolysis was measured for 3–5 min after addition of the enzyme. The activity was calculated from the slope of this curve and expressed in katal.

Enzyme. Two preparations of pure porcine pancreatic lipase (triacylglycerol acyl-hydrolase, EC 3.1.1.3.) were used during this study. They were a gift from Dr. M. Rovery (Marseilles, France) and were prepared as described in (5). Sample A, which was kept in concentrated solution (4.8 mg/mL), showed a lipase activity of 213 microkatal (μkat)/mg, whereas sample B, which was more diluted (1.49 mg/mL), had an activity of 84 μkat/mg: both activities were measured against tributyrylglycerol emulsion.

Determination of particle size of emulsified lipids: (i) triolein emulsion. The distribution of lipid droplet sizes was determined using the particle size distribution analyzer Capa-700 (Horiba, Kyoto, Japan). Capa-700 is based on a liquid-phase dispersive sedimentation method (gravity and centrifugal force) whereby measurements are made by means of an optical transmission method at 560 nm (photosedimentometry). The validity of the method has been evaluated recently (6), and the data obtained here with the Capa-700 were checked using calibrated microparticles in the size range 0.2–100 μm (polystyrene size standard kit; Polysciences, Warrington, PA). The mean discrepancy between the expected and measured median diameters of the microparticles was ±10.3%. Measurements were performed on aliquots of the triolein emulsion diluted in distilled water up to an optical density of between 0.5 and 1.0 at 560 nm. The results were expressed in the form of droplet size distributions. The median size values were calculated with the particle analyzer software from the droplet size distribution. By varying the mixing time of the oil–gum arabic-water mixture, a series of triolein emulsions in gum arabic were prepared. Increasing the mixing time from 30 s to 60 s, 120 s, 180 s, and 300 s decreased the median diameter of the lipid droplets from 1.75 μm to 1.67 μm, 1.24 μm, 1.08 μm and 0.84 μm, respectively. With mixing time of 30 s, the average median diameter of the lipidic droplets was found to be 1.84 μm ± 0.10 μm (n = 3). *(ii) tributyryl glycerol emulsion.* Due to its instability, tributyrylglycerol emulsion was not analyzed using the particle size distribution analyzer. The lipid emulsion was diluted ten times with water, and a droplet rapidly examined under a microscope and photographed. The average diameter of the smallest droplets, which were assumed not to have undergone any coalescence, was taken to be representative of the size of the oily phase during the assay: 30 ± 10 μm.

Recovery of unbound enzyme. The distribution of lipase between the aqueous and oily phases of tributyrylglycerol was determined as previously described by Borgström (7). In a lipase assay system, the enzyme was added to 25 mL of the aqueous phase containing 0.5 mL of tributyrylglycerol under stirring. After recording the hydrolysis for 5 min, the aqueous phase was aspirated with a syringe and filtered through a single-use hydrophilic filter unit with a porosity of 0.45 μm (Minisart NML, 2.5-cm diameter; Sartorius, Göttingen, Germany). The filtrate was then measured to determine the lipase activity.

Analysis of data. All of the calculations were performed on a personal computer. The experimental data were fitted to various equations using a Newton-Gauss algorithm (8). The quality of the fit was evaluated using standard methods, such as those described in the review by Mannervick (9).

RESULTS

Lipolytic activity as a function of the enzyme concentration. Figure 1A gives a classical plot of enzymatic activity as a function of the substrate concentration. The specific activity

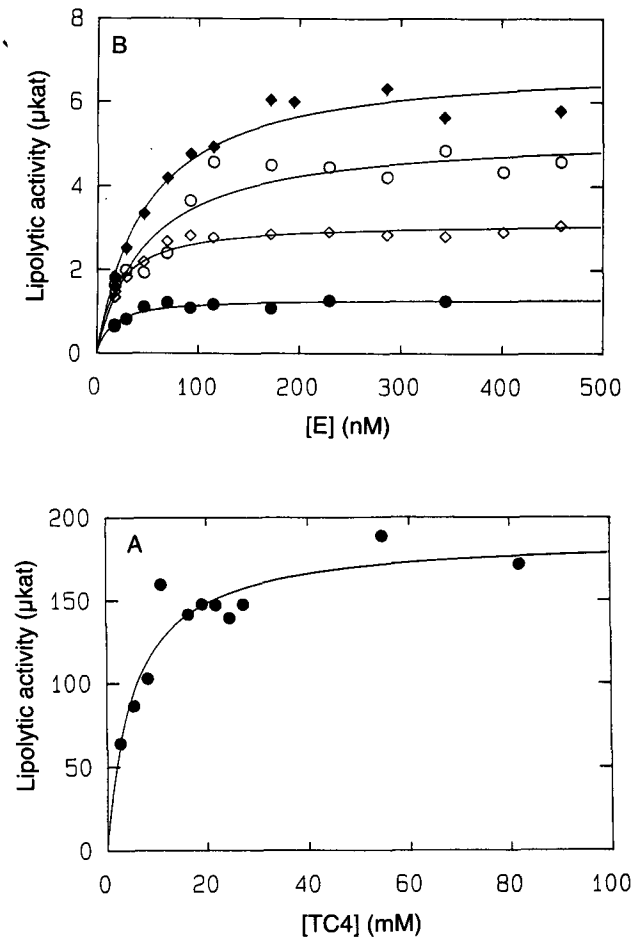


FIG. 1. (A) Lipolytic activity of porcine pancreatic lipase (2.2 μg), sample B, as a function of the tributyrylglycerol concentration [TC4], pH 8.0. Fitting the data to a hyperbola using a Newton-Gauss algorithm gave a V_{max} = 186 nkat, corresponding to a specific activity of 84 μkat/mg and a $K_{m\ apparent}$ = 5.4 mM. (B) Lipolytic activity of porcine pancreatic lipase, sample B, as a function of the concentration of added enzyme [E], with different amounts of tributyrylglycerol present in the assay, pH 8.0: ●, 125 μL; ◇, 250 μL; ○, 375 μL; ◆, 500 μL. Fitting the data to a hyperbola using a Newton-Gauss algorithm gave the following V_{max} values: 1.31, 3.14, 5.26, and 6.95 μkat as well as the $K_{m\ apparent}$ values: 15, 20, 50, and 47 nM (average value: 33 ± 18 nM).

of lipase, sample B, 4368 kat • mole^{-1}, was obtained after fitting the data to a hyperbola, and a $K_{m\ apparent}$ value of 5.4 mM was obtained.

Figure 1B gives the lipolytic activity of pancreatic lipase as a function of the enzyme concentration at the various tributylglycerol concentrations used in the assay. At small enzyme concentration values, the curve giving the lipolytic activity as a function of the enzyme concentration was almost linear for substrate concentrations over 250 µL, and the specific activity of the enzyme (kat/kg of protein) as well as the molar turnover (kat/mole of enzyme) could be calculated as usual. After adjusting the curves in Figure 1B to a hyperbola using the Newton-Gauss algorithm, the maximal lipolytic activity values were obtained at infinite enzyme concentrations at various substrate concentrations. Values of 1.31, 3.13, 5.26, and 6.94 µkat were obtained for 125, 250, 375, and 500 µL of tributyrylglycerol, respectively, added to the assay medium. In addition, the values corresponding to pseudo-K_m averaged to 33 ± 18 nM.

Assessment of the specific activity of pancreatic lipase at high enzyme concentrations. The adsorption of lipases to interfaces generally fits the Langmuir adsorption isotherm (1,10), which supports the fact that each molecule of lipase probably interacts with the interface independently of the others. The hypothesis was tested that every molecule of enzyme works independently, under conditions in which the lipidic interface is close to saturation with enzyme. Several assays were conducted with 2.4 µg (a catalytic amount far from the saturation conditions) and 200 µg of pancreatic lipase (an amount of enzyme leading to 80% saturation of the lipidic interface). These assays involved filtering the lipidic emulsion to differentiate between the enzyme bound to the oily phase, which was retained by the filter, from the free enzyme in the solution. The results are presented in Table 1. In those cases in which 2.4 µg (1.84 nM) of enzyme (138.7 µkat/mg) was used, 100% of the enzymatic activity was accounted for after filtering the lipid emulsion, whereas the bound enzyme ranged around 95%. In those cases in which 200 µg (154 nM) of enzyme (198.2 µkat/mg) was involved, the activity recovered accounted for 85% of the activity involved in the assay. Only 19% of the injected enzyme activity was measured under these assay conditions; 29.7% was found to be unbound. The identified losses amounted to 36.2%; they were attributed mainly to nonspecific adsorption during the filtration process and to the rapid decrease in specific activity of the enzyme stock solution after dilution. Within less than 10 min, a decrease from 198.2 to 125 µkat/mg was measured (data not shown). Assuming that all of the nonrecovered activity (15%) corresponded to adsorbed but nonactive enzyme, the specific activity of the adsorbed lipase must therefore have been at least [19/(19 + 15)]100 = 56% of that measured at the beginning of the saturation curve.

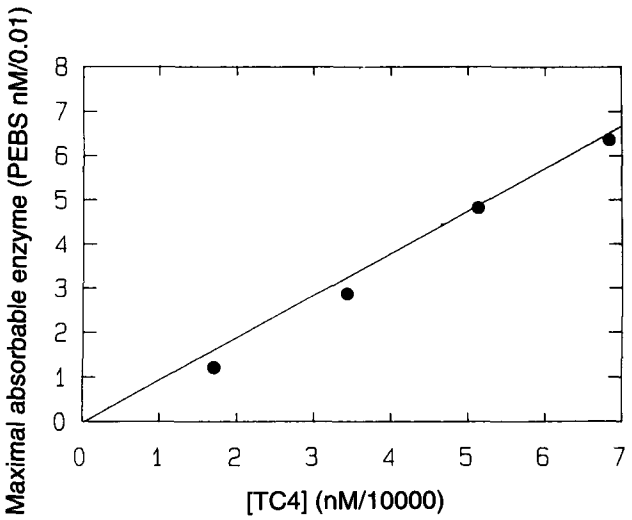


FIG. 2. Maximal adsorbable enzyme, expressed in protein (enzyme) binding site (PEBS) concentration, as a function of the substrate concentration [TC4]. The V_{max} values in Figure 1B were divided by the specific activity determined from Figure 1A leading to amounts of maximal adsorbable enzyme under the assay conditions, 25 mL, expressed in terms of PEBS concentration, which were plotted vs. the corresponding tributyrylglycerol concentrations. The data were fitted to a line forced through the origin: $y = \alpha$ [Tributyrylglycerol, M] + β; the fit gave: $\alpha = 0.957 \times 10^{-6}$ and $\beta = 2.17 \times 10^{-9}$; $r = 0.994$.

The protein (enzyme) binding site (PEBS). Assuming that every molecule of adsorbed enzyme works independently, it was possible to express the lipolytic activity in terms of the concentration of adsorbed lipase molecules. This was done by dividing the lipolytic activity per of volume unit (kat • volume^{-1}), taking into account the volume of the assay, by the molar turnover (kat • mole of enzyme^{-1}). Hence, the maximal values of the lipolytic activity measured in the presence of various concentrations of lipidic substrate, as obtained from Figure 1B, were transformed into a concentration of adsorbable molecules of lipase at the saturation of the lipidic interface. Figure 2 shows that abacus giving the maximal concen-

TABLE 1
Partition of Pancreatic Lipase Between the Lipidic and Aqueous Phases

Amount of enzyme added (total activity added)	Activity measured, nkat (% of total activity)	Activity recovered from the aqueous phase, nkat (% of total activity)	Identified losts, nkat (% of total activity)	Accounted activity, nkat (% of total activity)
2.4 µg (330 nkat)	323 ± 16 (96.8)	20.8 ± 7.5 (6.4)	3.7 (0.01)	347 (103.2)
200 µg (39600 nkat)	7600 (19.0)	11800 (29.7)	14400 (36.2)	33700 (85.0)

tration of adsorbable molecules of lipase, measured under our specific assay conditions, with respect to the concentration of tributyrylglycerol. A linear relationship between the concentration of substrate (mole · liter^{-1}) and the maximal concentration of adsorbable molecules of lipase (mole · liter^{-1}) ($y = \alpha$ [Tributyrylglycerol, M] + β, with $\alpha = 0.957 \times 10^{-6}$; $\beta = 2.17 \times 10^{-9}$ and $r = 0.994$) was observed. The slope of this curve (0.957×10^{-6}) indicates the efficiency of the assay system in terms of the "room" (mole · liter^{-1}) for lipase adsorption created by a concentration of one mole per liter of tributyrylglycerol. The inverse of the slope (1.04×10^{6}) gives the number of molecules of tributyrylglycerol needed to create the "room" for adsorbing one molecule of lipase. We propose to use the concept of PEBS as a means of expressing this "room" for the adsorption of one molecule of lipase in this particular case.

Using the abacus in Figure 2, we converted a lipid substrate concentration, reflecting only the proportions of the components of the heterogeneous mixture (x mL of lipidic phase + y mL of aqueous phase) into a PEBS concentration which can be used to express the true volumetric concentration of substrate (dimension: mole · liter^{-1}). Hence the kinetic data can be written as follows according to the Michaelis-Menten model:

$$\begin{aligned} &\text{Enzyme (solution)} + \text{PEBS (solution)} \xleftrightarrow{k1/k2} \\ &\text{Enzyme} \approx \text{PEBS (solution)} \xrightarrow{k3} \\ &\text{Enzyme} \approx \text{PEBS*} \text{ (solution)} + \text{Products} \end{aligned} \qquad [1]$$

This formula made it possible to use the PEBS concentration as if it stood for the concentration of the substrate in homogeneous catalysis, and thus to calculate a K_m value expressed as a volumic PEBS concentration. Table 2 gives the K_m values calculated in the case of porcine pancreatic lipase under various conditions involving tributyrylglycerol emulsion as substrate as well as a coarse emulsion of triolein in gum arabic and various pancreatic lipase preparations. The results ranged around 7.5 ± 2.0 nM PEBS ($n = 4$) regardless of the assay conditions (fast or slow stirring) when tributyrylglycerol was used as substrate, and 2.7 nM PEBS when triolein was used as the substrate. Very fine triolein emulsions were not suitable in this case because their hydrolysis was too fast and therefore not compatible with the capacities of the pHstat apparatus.

Calculation of the K_D. In the Michaelis-Menten model presented above, the species Enzyme ≈ PEBS and Enzyme ≈ PEBS*, differing, in our case, by a single molecule of tributyrylglycerol out of 1.04×10^{6} molecules, can be taken to be identical. For this reason, k3 presumably had a negligible effect on the equilibrium concentration of the Enzyme ≈ PEBS complex.

If one writes the initial concentrations of enzyme and PEBS as follows: $[E]_0$ and $[PEBS]_0$, and if at time t: [E] and [PEBS], assuming a stoichiometry of n between the enzyme and the PEBS (the value $n = 1$ was assumed at the beginning of the study), the concentration of free sites at time t, can be written as follows:

$$[\text{PEBS}] = n\,[\text{PEBS}]_0 - [\text{Enzyme} \approx \text{PEBS}] \qquad [2]$$

and:

$$K_D = \frac{[\text{E}]\,\{n[\text{PEBS}]_0 - [\text{Enzyme} \approx \text{PEBS}]\}}{[\text{Enzyme} \approx \text{PEBS}]} \qquad [3]$$

which gives

$$\frac{[\text{Enzyme} \approx \text{PEBS}]}{[\text{PEBS}]_0} = \frac{n[\text{E}]}{K_D + [\text{E}]} \qquad [4]$$

Figure 3A gives the data in Figure 1B, after transformation according to the abacus in Figure 2, and plotted to illustrate the latter relationship. After fitting the data using a Newton-Gauss algorithm, the values $K_D = 9.08 \pm 1.51$ nM and $n = 0.81 \pm 0.02$ were obtained. The same data were also plotted on a Scatchard plot. Figure 3B gives these results, which have been presented for convenience with a normalized abscissa. Values of $K_D = 9.03 \pm 2.66$ nM and $n = 0.84 \pm 0.10$ were obtained.

Determination of the surface PEBS density. Using the total area measurements obtained on the lipidic substrate as described in the Materials and Methods section, it was possible to calculate the superficial PEBS density (molecules · surface^{-1}) available for adsorption to pancreatic lipase molecules. As shown in Table 2 with lipase sample A, these values ranged between $10 \pm 6.2 \times 10^{11}$ and $14 \pm 0.7 \times 10^{11}$ molecules · cm^{-2} in the case of tributyrylglycerol–water interface and triolein–water interface, respectively, and plateaued at $4.5 \pm 2.7 \times 10^{11}$ molecules · cm^{-2} with lipase sample B.

TABLE 2
Pancreatic Lipase: Kinetic and Physicochemical Parameters

Substrate	$K_{m\ apparent}$ Triacylglycerol, mM	$K_{m\ apparent}$ Enzyme, nM	K_m PEBS, nM	K_d PEBS, nM	Maximal surface density PEBS, molecules · cm^{-2}
Tributyrylglycerol (lipase, sample A)[a]	29 ± 6	21	7.5 ± 2.0	—	$10.4 \pm 6.2 \times 10^{11}$
Tributyrylglycerol (lipase, sample B)[a]	5.4	—	7.5	—	$4.5 \pm 2.7 \times 10^{11}$
	—	33 ± 18	—	9.1 ± 1.5	—
Triolein, gum arabic (lipase, sample A)[a]	1.5	169	2.7	—	$14.0 \pm 0.7 \times 10^{11}$

[a]Enzymes; PEBS, protein (enzyme) binding site.

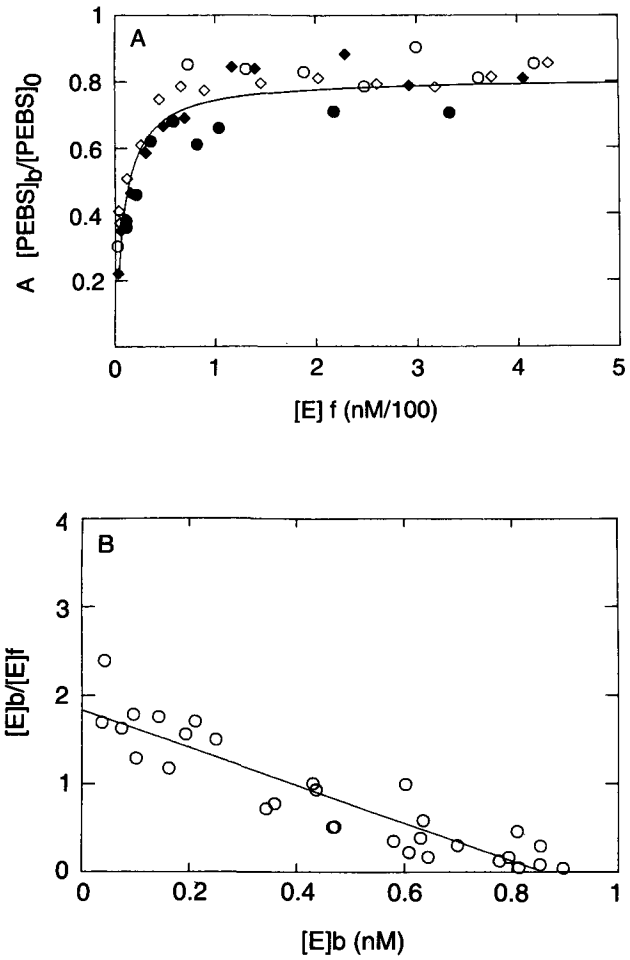


FIG. 3. (A) Plot of kinetic data from Figure 1B after transformation according to the abacus in Figure 2 in terms of the molar fraction of bond over initial PEBS species, $[PEBS]_b/[PEBS]_0$, as a function of the concentration of free enzyme, $[E]_f$. Fitting the data to a hyperbola using the Newton-Gauss algorithm gave: K_D = 9.08 ± 1.51 nM and n = 0.809 ± 0.022. (B) Scatchard plot of enzyme species after normalization of the abscissa for the various tributyrylglycerol concentrations used. K_D = 9.03 ± 2.66 nM and n = 0.844 ± 0.100. See Figure 2 for abbreviation.

DISCUSSION

The aim of the present study was to tackle the old problem of expressing the substrate concentration in heterogeneous catalysis, with special emphasis on lipolysis. Figure 1 and Table 2 show how ambiguous the determination of the kinetic constant K_m can be in heterogeneous catalysis, depending on the choice of the variable: the lipid substrate concentration or the enzyme concentration; values differing by six orders of magnitude were obtained for the same kinetic constant K_m of the same reaction. This strongly supports the need to use the qualitative term "apparent," which is often associated with the K_m values determined on the basis of lipid concentrations. A new concept is presented here for expressing the substrate concentration in heterogeneous systems such as those involved in the lipolysis of triacylglycerol emulsions by pancreatic lipase.

According to this concept, it is postulated that a stoichiometry of one exists between the protein (enzyme) that binds the lipidic interface and the "room" available for it to bind, which implies that under saturating conditions the concentration of bound protein (enzyme) is equal to the amount of "room." Hence, the amount of "room" existing in a heterogeneous system, under given conditions, can be calculated from the saturation curve of this system with the protein (enzyme) under study through the basic relation: one molecule of bound protein (enzyme) per liter corresponds to one unit of "room" per liter. This "room" or PEBS, to which no specific properties have been attributed, is taken to be the substrate involved in homogeneous catalysis.The interaction between the soluble protein (enzyme) and the lipidic emulsion is described in terms of an interaction with PEBS as follows:

$$\begin{aligned} &\text{Enzyme (solution)} + \text{PEBS (solution)} \xleftrightarrow{k1/k2} \\ &\text{Enzyme} \approx \text{PEBS (solution} \xrightarrow{k3} \\ &\text{Enzyme} \approx \text{PEBS* (solution)} + \text{Products} \end{aligned} \qquad [5]$$

The ratio between the PEBS concentration and that of the hydrophobic phase (substrate) present in a volume unit of the aqueous phase of the assay (a ratio between two volumetric concentrations of molecules, dimensionless) was evaluated. This ratio was given by the slope of the abacus of Figure 2, $\alpha = 0.957 \times 10^{-6}$. This value serves as an index to the efficiency of the assay system at binding the protein (enzyme) under study. Its inverse gives the number of molecules of hydrophobic phase (substrate) needed to bind one protein (enzyme) molecule, i.e., the number of molecules of lipid constituting an oligomer (PEBS). The smaller this number, the more efficient the hydrophobic phase in the binding of the protein will be. In our case, Enzyme ≈ PEBS is a complex consisting of one molecule of enzyme with 1.04×10^6 molecules of tributyrylglycerol, and Enzyme ≈ PEBS* is the same complex, containing one molecule less of tributyrylglycerol. Consequently, one may take the two enzymatic complexes Enzyme ≈ PEBS and Enzyme ≈ PEBS* to be identical and, in the K_m equation, neglect the term k3, so that it is possible to identify K_m and K_D. It is worth noting that the pseudo-K_m value of 33 ± 18 nM, as obtained from Figure 1B with pancreatic lipase, ranges around the protein (enzyme) concentrations values giving 50% saturation of the lipidic interface (K_D): 132 and 40 nM, for bovine serum albumin and β-lactoglobulin, respectively. as calculated from Reference 13. In addition, it is worth noting that the value of this pseudo-K_m (33 ± 18 nM) calculated for the enzyme ranged with the K_m calculated using the PEBS concentration (7.5 nM), conversely to that of the apparent K_m calculated for the triacylglycerol which ranged six orders of magnitude greater (29 ± 6 mM, Table 2).

According to this model, the PEBS should be viewed as an oligomer of lipid molecules devoid of cooperativity. In a way, this is the opposite situation to that of an oligomeric enzyme acting on a monomeric substrate in homogeneous catalysis. This transformation can be used to describe the binding

and kinetic data obtained on heterogeneous systems with the same formality as that used with homogeneous systems, and to calculate the Michaelis-Menten K_m values as well as the Scatchard K_D values in terms of the PEBS concentrations. It is worth mentioning that the PEBS concentration (molecules · volume^{-1}) takes into account both the "surface concentration" of the lipid emulsion (surface · volume^{-1}), a quantitative parameter, and the PEBS surface density (molecules · surface^{-1}) at saturation of the lipidic-water interface, which is a qualitative parameter, in the form of the product of these two factors. The PEBS surface density values obtained (see Table 2) were comparable to the maximal surface density values obtained with of human gastric lipase (10.6×10^{11} molecules · cm^2), bovine serum albumin (11×10^{11} molecules · cm^{-2}), and β-lactoglobulin (14×10^{11} molecules · cm^{-2}) adsorbed to a lipid monolayer of dicaprin at 35 dyn · cm^{-1} (11–13), as well as with porcine pancreatic lipase (29.5×10^{11} molecules · cm^{-2}) and porcine pancreatic carboxylester lipase (21×10^{11} molecules · cm^{-2}) obtained by a different technique on different lipid interfaces (14,15). In addition, the maximal superficial PEBS concentration value was found to amount to 20% of the surface coverage of the lipid interface, assuming that a lipase molecule occupies 2000 Å^2 at the interface.

According to Scatchard, the answer to complicated binding problems of this kind will depend on the answers to the simpler questions "How many?" and "How tightly bound?" (16). It is very likely that in our case the surface density of PEBS available for protein (enzyme) adsorption may answer the question "How many?", and that on the other hand, the K_D values calculated in terms of the PEBS concentration give the strength of the interaction and may answer the question "How tightly bound?", if one uses the relation: $\Delta G = -RT \text{ Log } K_D$ (10 kcal, in our case), thus opening the way to a thermodynamic approach to the interaction.

There exist many different ways of studying the adsorption of a protein to a lipidic interface. In the present study, the catalytic activity of pancreatic lipase was taken as an example for evaluating the maximal amount of protein capable of adsorbing to various lipidic emulsions. To transform a lipolytic activity into an amount of adsorbed enzyme, the assumption that the specific activity of the lipase was constant along the whole saturation curve had to be made. Table 1 shows data which are consistent with the conclusion that the specific activity of the enzyme was at least 56% of that measured at the beginning of the saturation curve, assuming that in the least favorable case, all of the unrecovered enzyme was bound, but that its activity was weak at the lipidic interface when almost 80% of the interface saturation was reached. If the specific activity of the lipase had not been constant along the whole saturation curve, this would have introduced an error in evaluating the maximal amount of bound enzyme. The magnitude of this error was assessed from its effects on the Scatchard plot, and a value of $n = 0.844$ was obtained, which is not very different from $n = 1$, the theoretical value; a clear-cut linear response was obtained (Fig. 3B). The effects of any changes in the specific activity of the lipase would therefore be very limited in our case. It is worth noting that this bias, which possibly affects the constancy of the specific activity of the enzyme when the ratio of enzyme to substrate was high, is also to be found in situations in which the enzymatic activity is recorded as a function of the substrate concentration, as shown in Figure 1A, at the low lipidic substrate concentrations. Furthermore, in the latter situation, a classical plot of the data according to Lineweaver and Burk (17) increases the bias, because the experimental points corresponding to the range of concentrations where the constancy of the specific activity of the enzyme is the least dependable, weigh heavily on the slope of the curve and hence on the value of the $K_{m\ apparent}$. On the other hand, determining the mass of adsorbed protein (enzyme) using physicochemical methods would have introduced another bias, because no proof is available when using this technique, that only the molecular species corresponding to completely intact protein (enzyme) is giving off a significant signal. This situation is illustrated in Table 2, where two different samples, A and B, of pure lipase, differing in their specific activity by a factor of 2.53 (213 μkat · mg^{-1} over 84 μkat · mg^{-1}), lead to two different surface densities of active enzyme differing by a factor of 2.31 (10.4×10^{11} molecules · cm^{-2} over 4.5×10^{11} molecules · cm^{-2}). These data are consistent with the possibility that the inactive or weakly active molecules of lipase included in the lipase sample B preparation may have adsorbed to the lipidic interface. Under these conditions, the surface density of the lipase molecule species in sample A or B is probably the same, but the molecules adsorbed in sample A will be 2.5 times more active than those in sample B.

The K_m (K_D) values expressed as a PEBS concentration ranged around 10 nM (Table 2). The lowest value was measured on triolein emulsion. These values differed by six orders of magnitude from the $K_{m\ apparent}$ determined using the classical method. It is interesting to make a parallel with the K_D values of the pancreatic lipase obtained in the presence of colipase adsorbed to a lipidic interface, which also ranged around nM (18–20). When expressed in terms of a PEBS concentration, the kinetic and binding data correlate well, therefore, with physical parameters measured using various other techniques. The use of the PEBS concept might help to achieve a deeper insight into protein–lipid interactions.

ACKNOWLEDGMENTS

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Stimulation of Microsomal Cholesterol Ester Hydrolase by Glucagon, Cyclic AMP Analogues, and Vasopressin in Isolated Rat Hepatocytes

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ABSTRACT: Short-term activation of microsomal cholesterol ester hydrolase by glucagon, cAMP analogues, and vasopressin in isolated rat hepatocytes is described. Glucagon led to a dose- and time-dependent activation of cholesteryl oleate hydrolysis, but values returned to basal levels within 120 min. Exposure of isolated hepatocytes to 0.5 mM concentrations of dibutyryl-cAMP or 8-[4-chlorophenylthio]-cAMP, or 25 μM forskolin caused persistent activation of cholesterol ester hydrolase activity after a lag period of 30 min. The three agents resulted in early marked intracellular accumulation of cAMP that declined progressively, and moderate and sustained reductions in the diacylglycerol content. The actions of glucagon on hepatocytes were inhibited by pretreatment of cells with 10 nM [8-arginine] vasopressin. Vasopressin elicited a consistent and sustained increase in cholesterol ester hydrolase activity and diacylglycerol without affecting cAMP while reducing the effect of glucagon on cAMP. Furthermore, the effects of glucagon and vasopressin on the activation of cholesterol ester hydrolase were not additive despite the similarity of their stimulation of diacylglycerol formation. Blockade of vasopressin-mediated activation of cholesterol ester hydrolase and diacylglycerol content were induced by excess prazosin. These data suggest that stimulation of microsomal cholesterol ester hydrolase in isolated liver cells may involve at least two signal transduction systems.

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Cholesterol esters in the liver are subject to a constant cycle of synthesis and hydrolysis reactions. Two neutral cholesterol ester hydrolases (EC 3.1.1.13.) (CEH) located in cytosol and microsomes (1–5) are responsible for the release of cholesterol from the cholesterol ester stores and, as such, are potential points for regulation of cholesterol homeostasis in the body. Whereas cytosolic CEH has been purified and is fairly well characterized (6–8), little information is available about the biochemistry, the physiological role, and the regulation of the microsomal form of this esterase. Microsomal CEH is thought to have a role in determining the partitioning of cholesterol toward the bile canaliculi or the bloodstream and in maintaining the balance between free and esterified cholesterol in endoplasmic reticulum. The activity of neutral microsomal CEH was found to be different from that of hormone-sensitive lipase (9), neutral retinyl ester hydrolase, and acid CEH also located in microsomes (10). Microsomal CEH from rat liver is located in the luminal side of the rough membranes (11), shows a circadian rhythm unrelated to the feeding status of animals (12), and is inhibited by a cytosolic protein (13). The cytosolic inhibitor was reported to be very active in the female liver and to respond to changes in the hormonal and the nutritional status of female rats (14).

Reversible protein phosphorylation has been implicated as a common mechanism for the short-term regulation of cholesterol metabolism. The inhibition of β-hydroxy-β-methylglutaryl-CoA reductase activity in its phosphorylated form is well established (15–17). Contradictory results have appeared about the regulation of acyl-CoA:cholesterol acyltransferase (18–21) and bile acid synthesis (22–24) by second messenger-mediated processes. Much evidence has accumulated supporting the concept that cytosolic CEH is activated by cAMP-dependent phosphorylation in steroidogenic and other nonhepatic tissues. Whether or not liver-soluble CEH is regulated by phosphorylation mechanisms is open to question. Evidence for activation of cytosolic CEH by cAMP- and Ca^{2+}-mediated processes has been presented in rat liver (6), while findings suggesting inactivation by phosphorylation were presented in hepatocyte suspensions (25). The evidence currently available for the involvement of second messengers in the regulation of the microsomal CEH is scant. An *in vitro* cell-free study from our laboratory has revealed marked stimulation of microsomal CEH by cAMP-dependent protein kinase or Ca^{2+}/calmodulin, whereas enzyme inactivation was dependent on phosphatase action (26). This led us to suggest for the first time that, in the rat liver, microsomal CEH may be regulated by reversible phosphorylation, with the phosphorylated form of the enzyme being the more active form. More recently, we found that okadaic acid, a potent and specific inhibitor of cellular protein phosphatases 1 and 2A, caused early and sustained inhibitions of microsomal CEH in hepatocyte suspensions (27). In addition, the inhibitory effect of

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Abbreviations: CEH, cholesterol ester hydrolase; 8-Cl-cAMP, 8-chloro-cAMP; CPT-cAMP, 8-[4-chlorophenylthio]-cAMP; DAG, diacylglycerol; db-cAMP, dibutyryl-cAMP.

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okadaic acid was found not to be related to the dibutyryl cAMP (db-cAMP)-promoted stimulation of esterase activity (27). Hoang *et al.* (21) reported stimulation of neutral CEH activity by cAMP analogues and glucagon in the post-mitochondrial supernatant of cultured hamster hepatocytes, but there are no systematic studies on the effects of these compounds on the neutral microsomal cholesterol ester hydrolysis in isolated rat hepatocytes.

The present study was aimed at determining whether changes in neutral microsomal CEH activity are related to changes in the cellular content of cAMP in isolated rat hepatocytes. We used the hormone glucagon, forskolin, a potent activator of adenylate cyclase, the cell-permeable cAMP analogues db-cAMP and 8-[4-chlorophenylthio]-cAMP (CPT-cAMP) to elevate the cAMP content in hepatocytes. Vasopressin pretreatment was used to reduce the subsequent glucagon-stimulated cAMP production. Further, we examined the effects of these interventions on the cellular content of 1,2-diacylglycerol (DAG), which is taken to be an indirect measure of the protein kinase C branch of the phospholipase C-dependent signal transduction pathway.

EXPERIMENTAL PROCEDURES

Chemicals. Cholesterol [1-^{14}C]oleate (59.5 mCi/mmol) and the cAMP radioimmunoassay kit were from Amersham (Aylesbury, Bucks, United Kingdom). Collagenase and CPT-cAMP were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Bovine serum albumin (essentially fatty acid-free), glucagon, [8-arginine], vasopressin, forskolin, prazosin, db-cAMP, and soybean trypsin inhibitor were obtained from Sigma Chemical Co. (St. Louis, MO). 8-Chloro-cAMP (8-Cl-cAMP) was kindly supplied by Dr. Y. S. Cho-Chung (National Cancer Institute, Bethesda, Maryland).

Isolation of hepatocytes and sampling for enzyme activity. Female Sprague-Dawley rats (180–200 g), housed in temperature- and light-controlled rooms and maintained on a standard diet *ad libitum* with free access to water, were used for the experiments at 0830. Hepatocytes were isolated by collagenase digestion as previously described (28). Cell viability was assessed by the Trypan blue exclusion test and by determining lactate dehydrogenase retention (29), both measurements showing routinely 90–95% viability. Liver cells (0.5 g) were suspended in Krebs-Henseleit solution, pH 7.4, containing 2.5 mM $CaCl_2$, 10 mM glucose, and 2.5% bovine serum albumin, to give a cell density of 4–5 $\times$ 10^6 cells/mL. Hepatocytes were maintained in suspension by rotary shaking and were incubated at 37°C for 30 min prior to addition of the desired compound. The cells were continuously gassed with O_2/CO_2 (19:1). Glucagon was added in 20 µL of 3 mM HCl and forskolin in pure dimethylsulfoxide at a 0.2% final concentration in the medium. All other compounds were dissolved in distilled water. The control cells received the solvent only. At the end of the incubation, two 1 mL aliquots containing 50 mg cells were taken, and the cell pellets were rapidly frozen in liquid N_2 and stored at –20°C until they were processed to measure cAMP and DAG. The remaining incubates (0.4 g cells in 8 mL medium) were diluted 2.5-fold in ice-cold 20 mM Tris-HCl buffer, pH 7.4, containing 200 mM sucrose, 50 mM NaF, and 10 mM EDTA, and then centrifuged. The cell pellets were homogenized in 4 vol of the above buffer and submitted to cell fractionation (1,000 $\times$ *g*, 10 min; 22,000 $\times$ *g*, 10 min; 105,000 $\times$ *g*, 60 min, twice). Microsomes were resuspended in 1 vol of 20 mM Tris–HCl buffer, pH 7.4, containing 250 mM sucrose, and further assayed for CEH activity.

Assay of CEH. Microsomal CEH activity was measured as described before (26). In brief, the assay mixture contained 0.3 mg of microsomal protein in a 0.4 mL solution containing 100 mM imidazole-HCl buffer, pH 6.8, and 40 nmol cholesterol [1-^{14}C]oleate (specific activity 6.25 mCi/mmol) presented as mixed micelles of cholesterol oleate, lecithin, and sodium taurocholate in a 1:4.4:2.5 molar proportion (30). The incubation was stopped and the labeled free fatty acids released were measured as described by Martínez *et al.* (26). One Unit of CEH corresponds to the hydrolysis of one pmol cholesterol oleate per min at 37°C.

Measurement of the cellular content of cAMP and DAG. The absolute mass of cAMP in hepatocytes was determined by a commercial radioimmunoassay kit in ethanolic extracts of the cells (50 mg cells in 0.5 mL of 1% 1N HCl in ethanol) conducted following the manufacturer's instructions. For measuring the cellular DAG level, lipids were extracted exhaustively twice (31), separated by thin-layer chromatography in *n*-heptane/diisopropyl ether/acetic acid (70:30:2, by vol) and charred (32). The DAG bands were quantified by densitometry with an image analyzer BioImage (Millipore, Milford, MA) using dioleylglycerol as standard.

Chemical and statistical analysis. The protein concentrations were determined by the dye-binding technique of Bradford (33) using bovine serum albumin as standard. Statistical significance of results was assessed by the unpaired Student's *t*-test.

RESULTS

The results in Table 1 show that exposure of isolated rat liver cells to glucagon led to moderate, though consistent, dose-dependent activations of microsomal CEH and, as expected, to increases in cellular cAMP. The temporal relationship between the activity of CEH in the microsomal fraction and the content of cAMP in hepatocytes exposed to 100 nM glucagon was analyzed. Figure 1 shows the parallel changes in these parameters. Glucagon caused an immediate elevation of enzyme activity, that remained enhanced for at least 60 min and returned to the basal value 120 min after the hormone addition (Fig. 1A). The extent of increase was consistently around 30%. Concomitantly, a 4-fold increase was seen in the cAMP concentration after a 5-min incubation with the hormone. The cAMP then declined gradually, and by 120 min no intracellu-

TABLE 1
Dose-Dependent Effect of Glucagon on the Microsomal Cholesterol Ester Hydrolase (CEH) Activity and on the Intracellular cAMP Content in Isolated Hepatocytes[a]

Glucagon (nM)	Microsomal CEH (% of control)	cAMP (% of control)
0	100 ± 2	100
1	96 ± 1	135
10	122 ± 3[b]	282
100	134 ± 2[c]	310
200	141 ± 7[c]	ND

[a]The hepatocytes were incubated for 10 min with glucagon, and samples were then collected to measure the microsomal CEH activity and the cellular cAMP content. Parameters are expressed as the percentage of the value found in the absence of glucagon (100%), which was 44.8 ± 0.94 Units/mg protein for CEH activity (mean ± SE of four independent experiments) and 132 pmol/g cell wet weight for cAMP (mean of two experiments). Statistical significance of the difference in values in each column with respect to the control is: [b]$P \leq 0.05$; [c]$P \leq 0.01$; ND, not determined.

lar accumulation of this cyclic nucleotide was detected (Fig. 1B).

The temporal relationship between the neutral microsomal CEH activity and the cAMP level was further investigated in hepatocytes exposed to two types of cAMP-elevating agents that act independently of the glucagon receptor(s): the cell-permeable cAMP analogues db-cAMP and CPT-cAMP and the potent and selective adenylate cyclase activator forskolin. The kinetics of the enzyme stimulation by the cAMP analogues were found to be different from that induced by glucagon, and they are plotted in Figure 2A. Increases in CEH activity were observed after an initial lag period of 30-min exposure to a 0.5 mM concentration of either analogue and activity remained stimulated substantially within the 120-min exposure tested. Figure 2B shows that both db-cAMP and CPT-cAMP led to a drastic increase in the cellular content of cAMP shortly after its addition, after which it declined gradually. The reported different rate of metabolism of these analogues (34) was reflected in the magnitude and pattern of stimulation of cAMP and CEH activity. CPT-cAMP induced cellular accumulation of cAMP to a lesser extent at the beginning, but the effect persisted for a longer period than that seen with db-cAMP, and a similar profile of activation was seen in CEH. The cell permeable compound 8-Cl-cAMP has been reported to bind specifically to the regulatory subunit of the cAMP-dependent protein kinase type II in cancer cell lines (35). We found that 8-Cl-cAMP raised microsomal CEH activity and the increase in enzyme activity exhibited the same temporal pattern to that seen with the other site-selective analogue CPT-cAMP (results not shown). Raising intracellular cAMP levels by direct stimulation of adenylate cyclase with the diterpene forskolin gave the maximum increase in cAMP (5-fold) within 10 min incubation, and there was also a lag of 30 min before any increase in microsomal CEH activity was detected (Fig. 3). Again, the change in cAMP preceeded that of the enzyme activity.

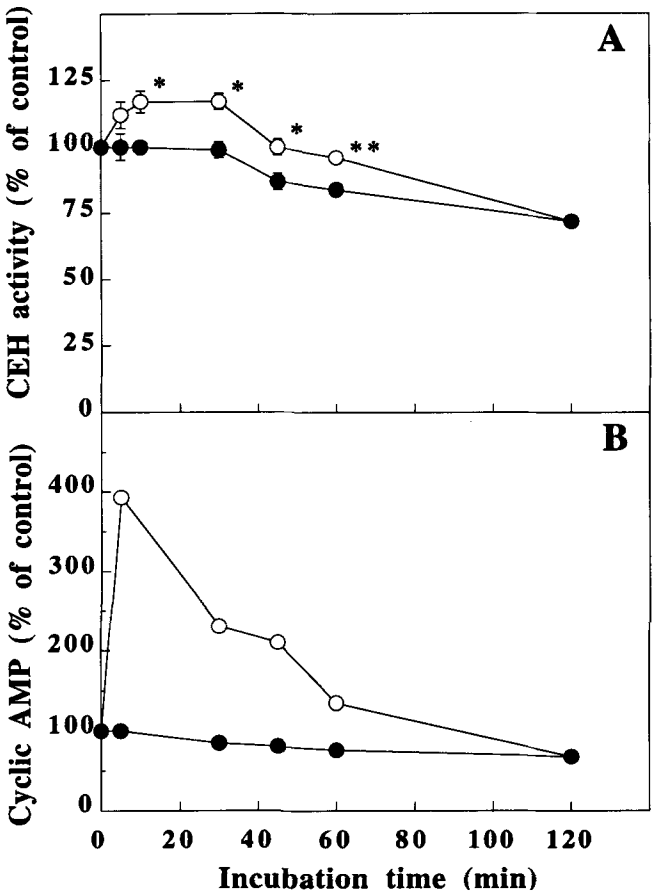


FIG. 1. Time-course of glucagon-mediated increases in microsomal cholesterol ester hydrolase (CEH) activity and in cellular cAMP content in isolated hepatocytes. The hepatocytes were incubated for 5 to 120 min with 100 nM glucagon (○), and the control cells (●) were exposed to the vehicle alone. Cell samples were collected at the indicated times and assayed for microsomal CEH activity (A) and cellular cAMP (B). Values shown are means ± SE of four different experiments for CEH activity and means of two for cAMP levels. Vertical bars not shown lie within the symbols. Parameters are expressed as the percentage of the value found in the control cells at the zero time (100%), which was 43.89 ± 2.49 Units/mg microsomal protein for CEH activity and 196 pmol/g cell wet weight for cAMP. Statistical significance of the difference in values with respect to the control is: *$P \leq 0.025$; **$P \leq 0.005$.

As a further analysis of the factors hypothetically relevant to the regulation of CEH activity in liver microsomes, we also measured the cellular concentration of DAG, one of the signals that mediate α-adrenergic responses *via* protein kinase C. Figure 4 shows changes in content of DAG in hepatocytes incubated over 5 to 120 min with the cAMP-elevating agents already mentioned: glucagon (100 nM) and forskolin (25 μM) in A, and db-cAMP (0.5 mM) and CPT-cAMP (0.5 mM) in B. The results show an overall decreasing trend. A gradual lowering of cellular DAG was caused by glucagon and forskolin. The response of this parameter to the cAMP analogues was similar in magnitude and shape. DAG were rapidly reduced and maintained between 25–30% below the

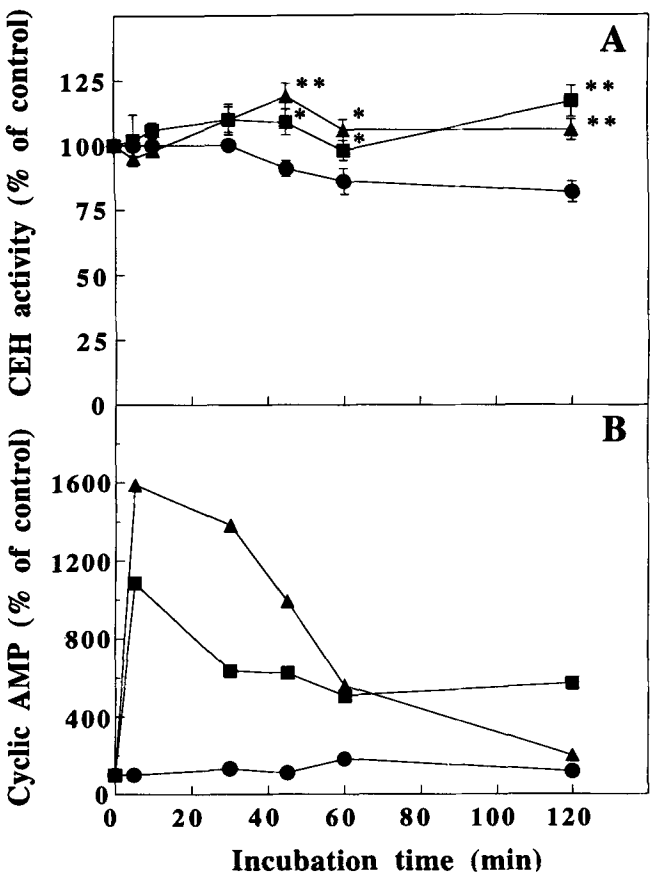


FIG. 2. Time-course of dibutyryl cAMP (db-cAMP)- or 8-[4-chlorophenylthio]-cAMP (CPT-cAMP)-mediated increases in microsomal cholesterol ester hydrolase (CEH) activity and in cellular cAMP content in isolated hepatocytes. The hepatocytes were incubated for 5 to 120 min with 0.5 mM db-cAMP (▲) or 0.5 mM CPT-cAMP (■), and the control cells (●) were exposed to the vehicle alone. Cell samples were collected at the indicated times and assayed for microsomal CEH activity (A) and cellular cAMP (B). Values shown are means ± SE of four different experiments for CEH activity and means of two for cAMP. Vertical bars not shown lie within the symbols. Parameters are expressed as the percentage of the value found in the control cells at the zero time (100%), which was 39.89 ± 2.11 Units/mg microsomal protein for CEH activity and 207 pmol/g cell wet weight for cAMP. Statistical significance of the difference in values with respect to the control is: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$, at least.

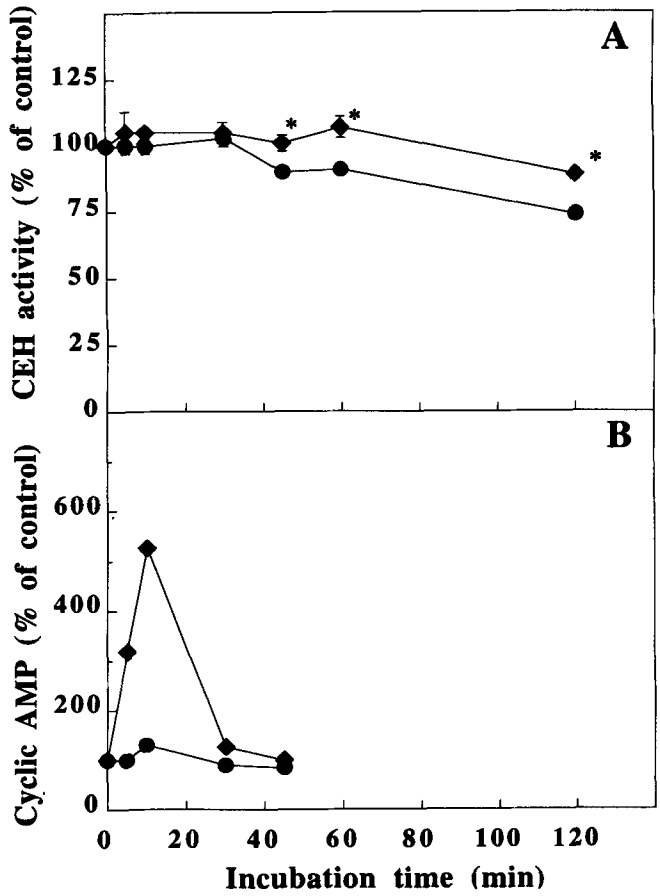


FIG. 3. Time-course of forskolin-mediated increases in microsomal cholesterol ester hydrolase (CEH) activity and in cellular cAMP content in isolated hepatocytes. The hepatocytes were incubated for 5 to 120 min with 25 mM forskolin (◆), and the control cells (●) were exposed to the vehicle alone. Cell samples were collected at the indicated times and assayed for microsomal CEH activity (A) and cellular cAMP (B). Values shown are means ± SE of four different experiments for CEH activity and means of two for cAMP. Vertical bars not shown lie within the symbols. Parameters are expressed as the percentage of the value found in the control cells at the zero time (100%), which was 45.89 ± 3.71 Units/mg microsomal protein for CEH activity and 188 pmol/g cell wet weight for cAMP. Statistical significance of the difference in values with respect to the control is: $^{*}P \leq 0.05$.

level in resting cells, except with a 60-min incubation, where DAG were essentially as they were in the controls.

Consistent with the fact that glucagon and β-adrenergic responses become desensitized by vasopressin exposure (36), Figure 5A shows that prior treatment of hepatocyte suspensions with 10 nM vasopressin for 5 min partially prevented the cAMP accumulation induced by 100 nM glucagon. The longer the cells were incubated with glucagon after vasopressin was added the less complete was the inhibition. Interestingly, exposure of cell suspensions to vasopressin alone gave a rapid and sustained activation of microsomal CEH without significantly affecting cAMP and, under these conditions, glucagon was unable to enhance further cholesterol esterase activity (Fig. 5B). Panel C of this figure shows the sustained 50–60% increase in cellular DAG induced by vasopressin either in the absence or in the presence of glucagon throughout the observation time. Though not shown, preincubation of cells with excess propranolol (150 μM) for 5 min did not produce any significant change in the concentration-dependent increase of CEH activity and cAMP level by glucagon.

Table 2 shows that simultaneous treatment of hepatocytes with both glucagon and vasopressin resulted in a nonadditive activation of CEH, an increase of cAMP lower than that due to glucagon, and an increase of DAG similar to that of vasopressin. Finally, the ability of the α_1-adrenergic agonist prazosin to modify vasopressin-mediated activation of CEH was examined. A 5-min pretreatment with excess prazosin (100

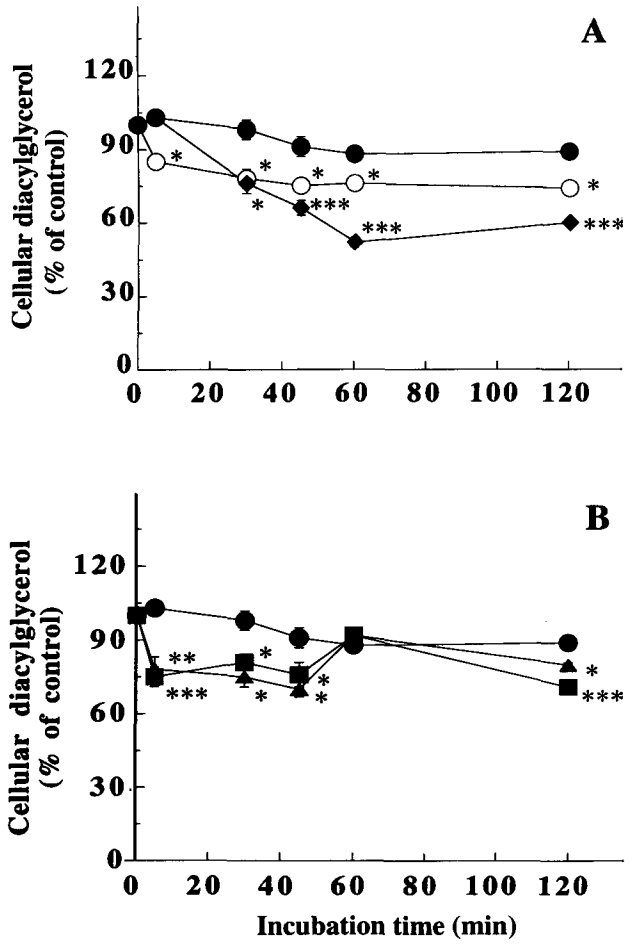


FIG. 4. Time-course of glucagon, forskolin, dibutyryl cAMP (db-cAMP), and 8-[4-chlorophenylthio]-cAMP (CPT-cAMP) to decrease the cellular content of diacylglycerol (DAG) in isolated hepatocytes. The hepatocytes were incubated for 5 to 120 min with 100 nM glucagon (○), 25 mM forskolin (◆), 0.5 mM db-cAMP (▲), or 0.5 mM CPT-cAMP (■). The control cells (●) were exposed to the vehicle alone. Cell samples were collected at the indicated times and assayed for DAG as described in the Experimental Procedures section. Values shown are means ± SE of four different experiments and are expressed as the percentage of the value found in the control cells at the zero time (100%), which was 4.12 ± 0.66 nmol/mg cell protein. Vertical bars not shown lie within the symbols. Statistical significance of the difference in values with respect to the control is: $*P \leq 0.025$; $**P \leq 0.01$; $***P \leq 0.005$.

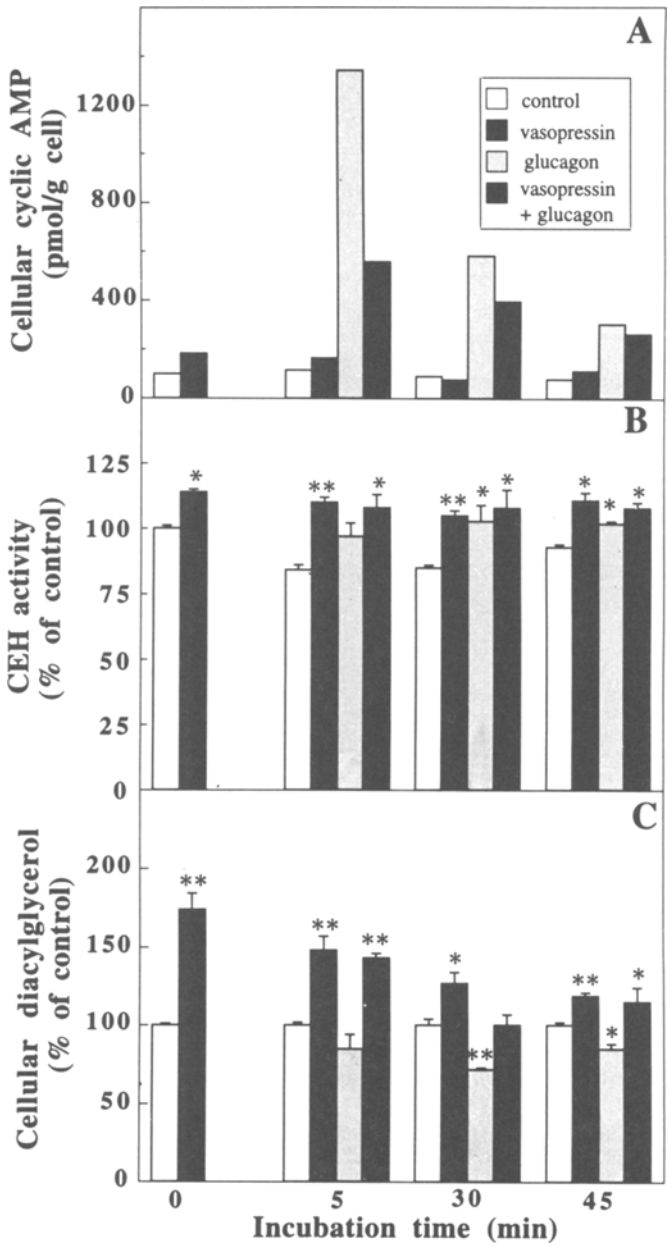


FIG. 5. Effect of prior exposure to vasopressin on glucagon-mediated changes in microsomal cholesterol ester hydrolase (CEH) activity and in cellular cAMP and diacylglycerol (DAG) levels. The hepatocytes were incubated for 5, 30, or 45 min with 100 nM glucagon subsequent to cell exposure to 10 nM vasopressin for 5 min. The control cells were pretreated with the vehicle alone. Cell samples were collected at the indicated times and assayed for microsomal CEH activity (A) and for cellular cAMP (B) and DAG content (C) as described in the Experimental Procedures section. Values shown are means ± SE of four different experiments except for cAMP (n = 2). Parameters are expressed as the percentage of the value found in the control cells at the 5 min time (100%), which was 40.9 ± 1.0 Units/mg microsomal protein for CEH activity and 4.36 ± 0.05 nmol/mg cell protein for DAG. Statistical significance of the difference in values with respect to the control is: $*P \leq 0.05$, minimun; $**P \leq 0.005$.

μM) was able to prevent vasopressin, alone or in combination with glucagon, from causing the increase in microsomal CEH activity, whereas activation of CEH by glucagon alone was still found. Table 2 also depicts that the changes in DAG due to the hormones were blocked by prazosin, while those in cAMP were unaffected.

DISCUSSION

The data presented herein provide evidence that exposure of rat hepatocytes in suspension to the hormones glucagon and/or vasopressin, or to compounds that increase intracellular cAMP, causes the rate of cholesterol oleate hydrolysis by microsomal CEH to be enhanced. The results suggest the involvement of several different mediators in this regulation. These findings agree with those we reported in a previous

TABLE 2
Effect of Prazosin on Glucagon and/or Vasopressin-Mediated Changes in the Microsomal Cholesterol Ester Hydrolase (CEH) Activity and in the Cellular cAMP and Diacylglycerol (DAG) Content in Isolated Hepatocytes[a]

	Microsomal CEH (Units/mg prot)	cAMP (pmol/g cell)	DAG (nmol/mg cell prot)
Control	41.61 ± 1.74	86	5.46 ± 0.37
Glucagon	56.58 ± 1.22[c]	232	4.64 ± 0.14[b]
Vasopressin	53.67 ± 2.00[b]	106	7.17 ± 0.16[b]
Glucagon + vasopressin	57.49 ± 1.35[c]	178	7.05 ± 0.88[b]
Prazosin	44.29 ± 1.94	119	5.51 ± 0.41
Prazosin + glucagon	49.70 ± 0.89[b]	265	4.82 ± 0.22
Prazosin + vasopressin	41.93 ± 0.44	132	5.77 ± 0.10
Prazosin + vasopressin + glucagon	38.84 ± 1.02	243	6.24 ± 0.39

[a]The hepatocytes were exposed to 100 μM prazosin for 5 min before addition of 100 nM glucagon and/or 10 nM vasopressin. After 30 min, cell samples were collected to measure the microsomal CEH activity and the intracellular cAMP and DAG content. Values shown are means ± SE of four independent experiments except for the measurement of cAMP (n = 2). Statistical significance of the difference in values in each column with respect to the control is: [b]$P \leq 0.05$; [c]$P \leq 0.005$. CEH activity in the presence of prazosin plus glucagon was significantly different ($P \leq 0.05$) from that in the presence of prazosin alone.

study with cell-free systems, in which stimulation of neutral microsomal CEH was seen after treatment of rat liver microsomes with a cAMP-dependent protein kinase or with Ca^{2+}/calmodulin (26), and with a recent report by Hoang *et al.* (21), indicating stimulation of neutral CEH activity by cAMP analogues and glucagon in the post-mitochondrial supernatant of cultured hamster hepatocytes.

We have found in this study with rat hepatocyte suspensions that the hormone glucagon, the cell permeable cAMP analogues db-cAMP, CPT-cAMP, and 8-Cl-cAMP, and the adenylate cyclase activator forskolin stimulate microsomal CEH activity. Comparison of the kinetics of CEH activity and cellular cAMP levels is consistent with a causal relationship. On one hand, the glucagon-induced stimulation in enzyme activity was approximately constant from 10 to 60 min of incubation, as was the induction of cAMP. Furthermore, increases in CEH activity and cAMP were parallel between 45 and 120 min after the addition of either cAMP analogue. However, at shorter incubation periods, when cAMP was at its maximum level, there was a lack of response in CEH activity. A similar temporal pattern was observed with forskolin, as the stimulation of the rate of cholesterol ester hydrolysis lagged behind the peak rise in cAMP. This was found to be the case for all the cAMP-elevating agents that act independently of the glucagon receptor(s). The effectiveness of the nonspecific cAMP analogue db-cAMP and the two site-selective analogues CPT-cAMP and 8-chloro-cAMP in activation of CEH were rather similar and higher than that of forskolin. As mentioned above, the greatest difference is that CPT-cAMP and 8-Cl-cAMP provoked marked activations at the longest incubation period tested. This may reflect different balances between penetration of the cell by each analogue, degradation within the cell and ability to activate target enzymes (37). These findings, taken together, suggest that cAMP may play a role in the stimulation of CEH activity. Whether or not cAMP exerts its effect *via* phosphorylation of the enzyme or by some other mechanism will be difficult to ascertain until the enzyme has been purified and can be studied at the molecular level.

In contrast to the cAMP-related agents discussed above, glucagon caused an immediate activation of CEH, suggesting that glucagon acts through a mechanism different from that of forskolin and cAMP analogues, at least in the initial stages of altering CEH activity. Glucagon is known to induce numerous actions in hepatocytes that appear to occur independently of any increase in cAMP and may be due to activation of lipid signalling pathways (36,38–41). Short-term effects of this hormone include stimulation of glycogenolysis, gluconeogenesis and fatty acid oxidation, and inhibition of fatty acid synthesis (42). In addition to glucagon (36,38–40), db-cAMP and the adenylate cyclase activator forskolin have also been reported to induce variations in the concentrations of cellular DAG and cytoplasmic free calcium (36,40,43). Our experiments show immediate reductions in DAG in cells treated with all the cAMP-elevating agents used in this work. Therefore, although it is likely that the effect of glucagon on CEH activity is mediated in part by increasing the intracellular concentration of cAMP, the change in the DAG content and the reported induction of calcium mobilization (36) may play a role in the early regulatory mechanism. This idea is further supported by the finding (not shown) that, although the glucagon-mediated increase in cAMP is potentiated by 3-isobutyl-1-methyl-xanthine, glucagon action on CEH is not affected by it. Conversely, when the glucagon-mediated increase in cAMP is reduced by pretreatment of hepatocytes with vasopressin, the glucagon action on CEH is not seen. Indeed, it is almost axiomatic to say that most receptors couple to multiple signalling pathways and that the product of one pathway can have feedback effects on the other signalling pathways, or activation of one receptor can modulate the response of another. The fact that the β-adrenergic blocker propranolol was without effect on glucagon actions, whereas vasopressin inhibited the ability of glucagon to stimulate cAMP content (36) and CEH activity, suggests that glucagon is acting *via* its own receptor(s).

In the liver, vasopressin is well known to produce marked changes in the phosphorylation state of diverse proteins through phosphoinositide-derived metabolites because hepatocytes contain a large number of vasopressin receptors, higher than those for angiotensin II or related Ca^{2+}-mobilizing hormones (44,45). Vasopressin treatment, alone, activated microsomal CEH activity without varying cAMP. The elevation in intracellular DAG found in vasopressin-treated hepatocytes was maintained when glucagon was added simultaneously. The magnitude and the time-dependence of vasopressin-induced DAG accumulation were in the range of previously reported data (44). In this situation, CEH remained activated at its maximum extent (about 35%), but there were

no observed synergistic effects. In addition, excess prazosin prevented the activation of CEH elicited by vasopressin, as expected from a role for DAG/Ca^{2+} in the activation. This indicates that the neutral microsomal CEH is consistently activated by occupancy of not only glucagon but also vasopressin receptors. It is not clear why there is no activation of CEH in cells exposed to vasopressin and glucagon following prazosin treatment. Under these conditions, it is assumed that prazosin is preventing vasopressin but not glucagon from activating CEH. Thus, whereas the increases in CEH activity and cAMP levels are seen, the glucagon-mediated decrease in DAG is abolished by prazosin. It is possible that the binding of glucagon to a DAG/Ca^{2+}-related receptor is disturbed by the α_1-adrenergic agonist, which in turn could affect in some way the response of CEH to the cAMP signal.

It is of interest to note that the magnitude of the effects on the neutral microsomal CEH activity caused by phosphorylating agents in the intact hepatocyte is much smaller than those found in cell-free systems (26). As a matter of fact, large effects on the microsomal cholesterol esterase, unlike the cytosolic CEH, are often not seen in studies with intact animals or isolated liver cells. A possible explanation is that microsomal CEH, as other enzymes involved in cholesterol homeostasis, is controlled by a variety of mechanisms besides phosphorylation that may be interconnected or acting simultaneously, this having the power to attenuate the effects of phosphorylation. Neither can we rule out that microsomal CEH has a maximum level for activation, once the phosphorylation cascades are switched on by occupancy of glucagon and/or vasopressin receptors. Whether the phosphorylation of an enzyme *in vivo* is finally expressed as a change of its activity depends on the concentration of substrate and effectors within the cell (46), parameters that can be altered when cells are exposed to agonists.

This study provides the first evidence that triggering the phosphorylation cascades by cAMP-dependent and Ca^{2+}-linked hormones in isolated rat hepatocytes correlates with increased activity of the neutral microsomal CEH. Whether the changes in this esterase activity have a real impact in the cholesterol flux inside the hepatocyte is presently under study.

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A Protein Tyrosine Kinase Associated with the ATP-Dependent Inactivation of Adipose Diacylglycerol Acyltransferase

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ABSTRACT: The activity that has been previously reported to reversibly inactivate adipose glycerolphosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT) *in vitro* in the presence of ATP is shown here to be partially purified from adipose tissue with an apparent molecular weight of 68 kDa. The activity responsible for inactivating DGAT is associated with a kinase activity as determined by phosphate incorporation both into microsomal proteins and into a synthetic tyrosine-containing peptide as substrate for protein tyrosine kinase. Two microsomal polypeptides of 53 and 69 kDa are major substrates of this kinase. Both DGAT inactivating and kinase activities assayed from the purified sample have been found to be insensitive to the Ser/Thr kinase inhibitor H-7 while being sensitive to genistein and tyrphostin-25. A crude protein phosphatase preparation from liver was capable of reversing the effects of both activities. The purified sample was also shown to inactivate GPAT in the presence of ATP. These results suggest that a protein tyrosine kinase, in concert with a protein tyrosine phosphatase, may regulate the activities of DGAT and GPAT by a phosphorylation–dephosphorylation mechanism.
Lipids 31, 277–283 (1996).

Simultaneous triacylglycerol synthesis and lipolysis allows adipose tissue to rapidly respond to changes in peripheral tissue requirements for fatty acids. Moreover, phospholipids and diacylglycerols are essential components of cell membranes and are mediators of hormone action. However, the regulation of glycerolipid synthesis is not yet well understood. There have been conflicting reports about the regulatory roles of hepatic diacylglycerol acyltransferase (DGAT) and phosphatidate phosphatase (PAP) (1–3). At present, a rate-limiting step in adipocyte triacylglycerol (TAG) synthesis has not been established. Rather, all enzymes appear to be functioning below saturation to allow multistep regulation. In this way, the steady-state triglyceride synthesis could increase without a rise in levels of phosphatidate and lysophosphatidate, which can potentially pose a threat to membrane stability and function (4).

Although it is well established that fatty acid incorporation into adipose tissue triglycerides is under hormonal control (4), the precise molecular events involved in the regulation of synthesis have yet to be elucidated. Whereas only glycerolphosphate acyltransferase (GPAT) has been found to be activated by insulin (5), catecholamines have been previously reported to decrease the activity of GPAT (6), PAP (7), and DGAT (6). However, in a more recent report (8), it was proposed that norepinephrine does not directly regulate TAG synthesis in adipocytes. *In vitro* experiments suggest that TAG synthetic enzymes, including GPAT (9,10), PAP (11), and DGAT (12,13) from various tissues, may be regulated by a phosphorylation-dephosphorylation mechanism. However, different kinases have been implicated with conflicting reports on their effects on the enzymes (11–16).

We have previously described the presence of an enzyme activity in adipose tissue which, in the presence of ATP, reversibly inactivates GPAT (17) and DGAT (18). We have also shown that this activity could not be replaced by different preparations of protein kinase C (PKC) and protein kinase A (PKA) (18). In the present report, we describe the partial purification of this enzyme activity from the adipose cytosol and demonstrate its association with a protein tyrosine kinase activity.

MATERIALS AND METHODS

Materials. Oleic acid, oleoyl-CoA, diolein (1,2-dioleoyl-*sn*-glycerol), L-glycerol-3-phosphate, ATP, CoA, DL-dithiothreitol (DTT), ethylenediaminetetraacetate (EDTA), fatty acid-free bovine serum albumin (BSA), 4′,5,7-trihydroxyisoflavone (genistein), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7), [3,4,5-trihydroxybenzylidene]-malononitrile (tyrphostin-25), tyrosine kinase synthetic substrate peptide (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly), Reactive Blue 2-Sepharose, S-Sepharose, Sephacryl S-200, DEAE-Sepharose CL-6B, polyethylene glycol compound (MW 15,000–20,000), SDS, acrylamide, Brilliant Blue G colloidal, and molecular weight standards were obtained from the Sigma Chemical Co. (St. Louis, MO). Filters (0.22 μm pore size) were obtained from Millipore (Bedford, MA). Phospho-

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Abbreviations: BSA, bovine serum albumin; DGAT, diacylglycerol acyltransferase; DTT, DL-dithiothreitol; GPAT, glycerolphosphate acyltransferase; PAGE, polyacrylamide gel electrophoresis; PAP, phosphatidate phosphatase; PKA, protein kinase A; PKC, protein kinase C; TAG, triacylglycerol; TED buffer, 50 mM Tris-HCl (pH 7.5)/1 mM EDTA/2 mM DTT; TMD buffer, 50 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$/1 mM DTT.

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cellulose paper (Whatman P-81) was obtained from Canadawide Scientific (Ottawa, Ontario, Canada). [9,10-^{3}H]Oleic acid (7.4 Ci/mmol) and L-[2-^{3}H]glycerol-3-phosphate (10.6 Ci/nmol) were obtained from Du Pont Canada Inc. (Mississauga, Ontario, Canada). [9,10-^{3}H]Oleoyl-CoA (100 Ci/mol) was prepared from the radioactive fatty acid following the method of Bishop and Hajra (19) as previously described (20). ^{32}P-γ-ATP (3 Ci/mmol) was obtained from Amersham (Oakland, Ontario, Canada). Universol (liquid scintillation cocktail) was from ICN Biomedicals (Mississauga, Ontario, Canada).

Preparation of microsomes. Epididymal adipose tissue microsomes from male Sprague Dawley rats (Charles River Canada Inc., St. Constant, Quebec, Canada) were prepared by differential centrifugation of the homogenized tissue as previously described (17). When indicated, to remove any endogenous kinase, the microsomes were washed with a buffer containing 200 mM Tris-HCl (pH 9.0), 1 M NaCl, 1 mM EDTA, 0.25 M sucrose, and 1 mM DTT. Amounts of microsomes used in the experiments are indicated throughout this paper as the mass of microsomal protein.

Purification of DGAT inactivating activity. The activity responsible for DGAT inactivation was purified by fractionation of 50 mL of the 100,000 × *g* supernatant from homogenized adipose tissue, with the fractions of the purification sequence being tested for DGAT inactivation with washed microsomes (8 μg) as shown below. The cytosol was first mixed with solid ammonium sulfate to reach 55% ammonium sulfate saturation and the mixture was stirred at 4°C for one hour. After centrifugation the pellet containing the DGAT inactivating activity was resuspended in TED buffer [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 2 mM DTT] and applied to a 20 mL Blue-Sepharose column followed by elution with TED buffer containing 0, 0.3, and 1 M NaCl. The protein peak which eluted at 0.3 M NaCl contained the higher capacity for DGAT inactivation. It was dialyzed against TED buffer and applied to a 20 mL S-Sepharose column, which was eluted with 0.5 M NaCl in TED buffer. The pooled fractions containing the activity were dialyzed against TED buffer, applied to a 7 mL DEAE Sepharose column and eluted with 1 M NaCl in TED buffer. Fractions containing the activity were pooled, concentrated with polyethylene glycol (Sigma) and applied to a 45 cm Sephacryl S-200 column equilibrated with TED buffer. The final pooled fractions containing the activity were made to 20% (vol/vol) with respect to glycerol and stored at –20°C.

Protein phosphatase preparation. Protein phosphatase was partially purified from rat liver up to the ethanol precipitation step of the method of Brant *et al.* (21) as previously described (17).

GPAT assay. GPAT assay was performed at 37°C in a final volume of 0.5 mL containing 0.1 M Tris-HCl (pH 7.5), 2 mM DTT, 10 mM $MgCl_2$, 25 μM BSA, 0.1 mM oleoyl-CoA, 1 mM [^{3}H]glycerol-3-phosphate (1.2 Ci/mol), and microsomes (5–10 μg). After a 4-min incubation, the reaction was stopped by vortexing with 1 mL of butanol saturated with water. The butanol phase was washed three times with 1.5 mL of water saturated with butanol, and a 0.5 mL aliquot was counted in 7 mL Universol in a beta counter.

DGAT assay. DGAT was assayed as previously described (18) with minor changes by incubating 5–10 μg of microsomes for 5 min at 37°C in 0.5 mL of Tris-HCl (pH 7.5) containing 1 mM DTT, 10 mM $MgCl_2$, 5 μM BSA, 20 μM [^{3}H]oleoyl-CoA (7.5 Ci/mol), and 150 μM 1,2-diolein (delivered in 5 μL acetone). The reaction was stopped with 1.5 mL of isopropanol/hexane (1:1, vol/vol), and the hexane phase was washed three times with 0.75 mL of isopropanol/hexane (4:1, vol/vol) and 0.75 mL of 0.05% KOH. A 0.75-mL amount from the hexane phase was counted in 7.5 mL Universol in a beta counter.

DGAT/GPAT inactivating activity assay. ATP-dependent GPAT and DGAT inactivation was determined by performing the corresponding enzyme assays with 8 μg of microsomes (unless otherwise mentioned) and the indicated sample, in the presence and absence of 0.5 mM ATP. Inactivating activity was expressed as the percentage decrease in the rate of DGAT or GPAT activity caused by the addition of ATP as previously described (17,18). Since no linear dependence of DGAT/GPAT inactivation with time could be obtained in most of our assay conditions (18), our measurements of DGAT or GPAT inactivation are not rates but rather levels of inactivation which, in most cases, correspond to the maximum level observable with our microsomes preparation.

Protein kinase assay. Kinase activity was assayed by measuring the incorporation of ^{32}P from ^{32}P-γ-ATP into either microsomes or the tyrosine kinase synthetic-peptide substrate. Phosphorylation of microsomes was carried out in 0.1 mL of TMD buffer [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT] containing 0.5 mM ^{32}P-γ-ATP (40 Ci/mol) and 30 μg of microsomes. The reaction was initiated by addition of the kinase sample or, in the case of microsomes containing the endogenous kinase, by the addition of the ^{32}P-γ-ATP. After a 5 min incubation at 37°C, a 75 μL aliquot of the incubation mixture was applied to a 0.22-μm pore size filter (2-cm diameter) which was prewashed with 0.5 mL of 50 mM phosphate. The reaction was terminated by passing 2.5 mL of 50 mM phosphate through the filter disc under suction. Discs were dried and counted in 7 mL of Universol (ICN).

Phosphorylation of the synthetic peptide was carried out as described by Pike *et al.* (22) with some minor modifications. The incubation was carried out at 37°C in 20 μL of TMD buffer containing 1 mM synthetic peptide and 0.5 mM ^{32}P-γ-ATP (40 Ci/mol). The reaction was initiated by the addition of the kinase sample and terminated after five minutes by the addition of 30 μL of 5% trichloroacetic acid. After centrifugation, the supernatant was blotted on phosphocellulose paper (2 cm^2) and washed three times with 75 mM phosphoric acid. After drying, the paper was counted in 7 mL of Universol.

Protein assay. Protein concentrations were measured by the dye-binding assay of Bradford (24) using bovine serum albumin as the standard.

Electrophoresis. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out in a mini-protein gel electrophoresis apparatus (Bio-Rad), under reducing conditions using the discontinuous system of Laemmli (23), with 7.5 and 4.5% acrylamide resolving and stacking gels, respectively. Gels were stained with Brilliant Blue G colloidal (Sigma), dried and, when indicated, exposed to Fuji NIF-RX X-ray film. The following molecular weight markers (Sigma) were used: carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase b (97.4 kDa), and β-galactosidase (116 kDa).

RESULTS

Incubation of microsomes from rat adipose tissue with 0.5 mM ATP resulted in the partial inactivation of DGAT (33 ± 1%) as shown in Figure 1. These microsomes also contained an endogenous kinase activity that catalyzed the incorporation of ^{32}P from ^{32}P-γ-ATP into microsomes. Washing the microsomes with a high salt, high pH buffer caused the removal of both the ATP-dependent DGAT inactivating activity and the kinase activity (Fig. 1). The adipose tissue cytosolic fraction was able to restore both activities when added to the washed microsomes (Fig. 1), which is in agreement with previous results showing that the cytosol stimulated the ATP-dependent inactivation of microsomal DGAT (18).

Partial purification of the DGAT inactivating activity. The activity responsible for the ATP-dependent inactivation of DGAT was partially purified from the cytosol by a sequence of steps involving protein precipitation with ammonium sulfate as well as Blue Sepharose, S-Sepharose, DEAE-Sepharose, and Sephacryl S-200 chromatographies, as described in the Materials and Methods section. The activity bound to Blue Sepharose, S-Sepharose, and DEAE-Sepharose and coeluted with the protein peaks at NaCl concentrations of 0.3, 0.5, and 1.0 M, respectively (Fig. 2). In the case of the gel filtration column, the protein eluted in two overlapping peaks corresponding to 82 and 44 kDa, with the activity centered at 67 kDa (Fig. 2). Aliquots from the pooled fractions containing 1.2 μg of protein caused 46 ± 4% inactivation of DGAT in the presence of ATP. The final fraction containing the activity represented 0.1% of the starting protein (Table 1). SDS–PAGE of the purified fraction showed a major band at about 68 kDa (Fig. 3).

Phosphorylation of a tyrosine containing peptide. Consistently through the purification steps, the pooled fractions with DGAT inactivating activity were found to phosphorylate the synthetic peptide Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly (Table 1) which is a specific substrate of protein tyrosine kinases (22). Thus, a protein tyrosine kinase activity seemed to co-purify with the ATP-dependent DGAT inactivating activity. About 90% of the total cytosolic tyrosine kinase activity was lost at the salt precipitation step resulting in a 50% drop in specific activity. However, more than 50% of the remaining activity was recovered, with a purification factor of about 120-fold, through the last four chromatographic steps (Table 1).

Our purified kinase was also able to phosphorylate microsomes at a rate of 0.24 ± 0.08 μmol Pi · min^{-1} · mg^{-1}, which represents a 400-fold increase over the cytosolic specific activity (Table 1).

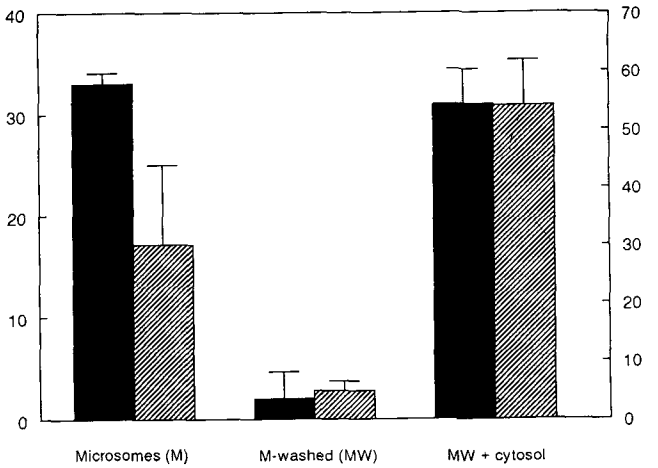


FIG. 1. Localization of the activity responsible for diacylglycerol acyltransferase (DGAT) inactivation. Microsomes (M) and washed microsomes (MW) were assayed for ATP-dependent DGAT inactivation in the presence and absence of cytosol (120 μg). For microsomal phosphorylation, 30 μg of microsomes with and without 60 μg cytosol were assayed for the incorporation of ^{32}P-phosphate from ^{32}P-γ-ATP into microsomes. Results are given as mean ± SEM (n = 5). Solid bar, ATP-dependent DGAT inactivation (%); ruled bar, microsomal phosphorylation (pmol Pi/min).

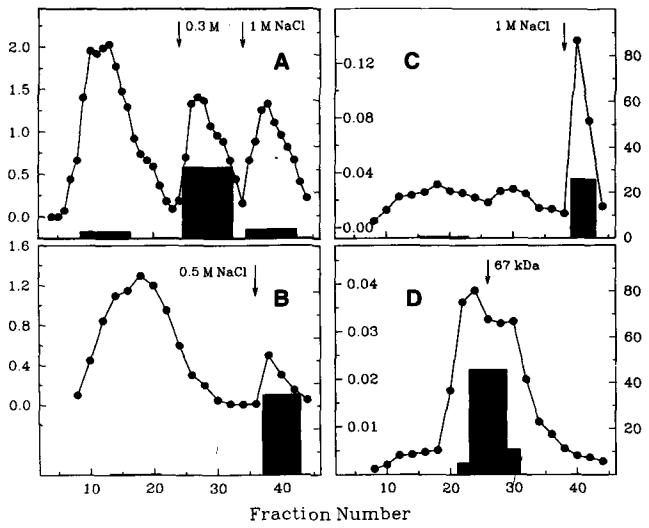


FIG. 2. Purification of DGAT inactivating activity. Elution profiles for the Blue Sepharose column (a), the S-Sepharose column (b), the DEAE-Sepharose column (c), and the Sephacryl S-200 column (d) are given (from Sigma Chemical Co., St. Louis, MO). Fractions were assayed for protein content. Pools were made of the protein peaks, as indicated by the bars, and 50 μL aliquots were assayed for DGAT inactivating activity. Fraction volume was either 1 mL (A) or 0.5 mL (B, C, and D). ●, Protein concentration (mg/mL); ■, DGAT inactivation (%). Abbreviation as in Figure 1.

TABLE 1
Purification Sequence of the Kinase Associated with DGAT Inactivation[a]

Purification step[b]	Total protein (mg)	DGAT inactivating activity	Microsome phosphorylation (nmol/mg/min)	Tyrosine kinase activity (nmol/mg/min)
Cytosol	150.0	+	0.62 ± 0.1 (4)	0.20 ± 0.03 (8)
Ammonium sulfate	37.5	+	n.d.[c]	0.10
Blue Sepharose	9.38	+	n.d.	0.56
S-Sepharose	0.57	+	n.d.	3.52
DEAE-Sepharose	0.29	+	n.d.	6.36
Sephacryl S-200	0.14	+	240 ± 80 (4)	11.7 ± 0.4 (8)

[a]Pooled fractions testing positive for the presence of diacylglycerol acyltransferase (DGAT) inactivating activity (Fig. 2) were assayed for protein content, for microsome phosphorylation, and for kinase activity with the tyrosine containing peptide. Where indicated, values given are means ± SEM (number of determinations).
[b]All from Sigma Chemical Co. (St. Louis, MO).
[c]n.d., Not determined

Effect on GPAT. Since we have previously shown that microsomal GPAT is also reversibly inactivated by an ATP-dependent activity in rat adipose cytosol (17), we determined whether or not the activity purified from the cytosol could inactivate GPAT *in vitro*. Addition of 1.2 µg of partially purified kinase preparation and 0.5 mM ATP to the incubation mixture of the GPAT assay resulted in 34 ± 3% inactivation, which is similar to the 38 ± 5% GPAT inactivation obtained with 120 µg of cytosol.

Effect of various protein kinase inhibitors. We have previously shown that DGAT inactivating activity in the cytosol could be inhibited by EDTA, but it was insensitive to inhibitors of serine/threonine kinases such as H7 and the protein inhibitor of PKA (18). We have now found that these two latter inhibitors decreased the level of microsome phosphorylation with cytosol by nearly 50% (data not shown), suggesting the presence of cytosolic serine/threonine kinases acting on microsomal proteins unrelated to DGAT activity. As shown in Figure 4, our kinase preparation showed a relatively lower sensitivity to inhibition of microsome phosphorylation by H7, indicating that a good part of the cytosolic serine kinase activity had been removed. By contrast, two tyrosine kinase inhibitors, tyrphostin-25 and genistein, were effective in blocking both the microsome phosphorylation and the DGAT inactivating activities of our purified fraction (Fig. 4).

Phosphorylation of microsomal proteins. Figure 5 shows the autoradiograph of ^{32}P-phosphorylated microsomal proteins run on SDS–PAGE. Two major bands at 53 ± 1.3 and 69 ± 1.1 kDa (four gels) were visible, as well as a third one at the top of the resolving gel which may represent an aggregate of proteins (lane 1). Other minor bands were also apparent in

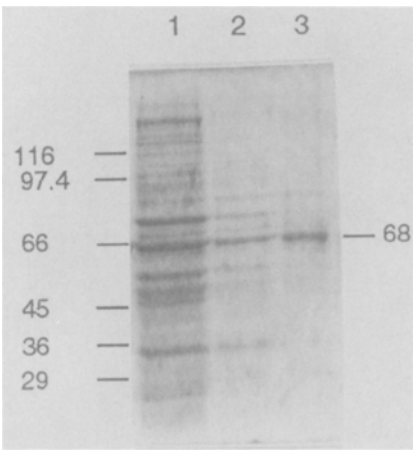


FIG. 3. Electrophoresis of the purified activity. SDS–polyacrylamide gel electrophoresis was run with 80 µg of the 55% ammonium sulfate pellet (lane 1), 20 µg of the 0.3 M NaCl pool from Blue Sepharose column (lane 2), and 10 µg of the Sephacryl S-200 pool (lane 3). The gel stained for protein with Brilliant Blue G colloidal. The molecular weight markers (kDa) (left) and the purified protein (right) are indicated. Company source as in Figure 2.

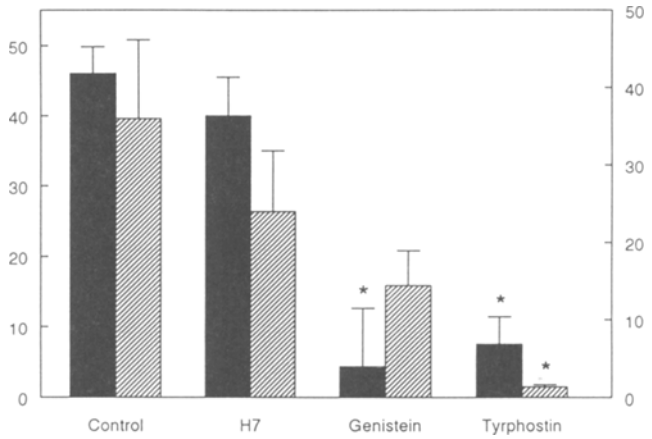


FIG. 4. Effect of kinase inhibitors on the purified activity. Washed microsomes (8 µg) were assayed for DGAT inactivation and phosphorylation of microsomes in the presence of the purified activity (2.4 µg/mL) and 0.2 mM of different inhibitors (H7, genistein, and tyrphostin-25). Results are given as mean ± SEM (n = 4). Significant inhibition ($P < 0.05$) was estimated by a paired *t*-test with the control (no inhibitor) as reference, and is indicated by *. Solid bar, ATP-dependent DGAT inactivation (%); ruled bar, microsomal phosphorylation (pmol Pi/min). Abbreviation as in Figure 1.

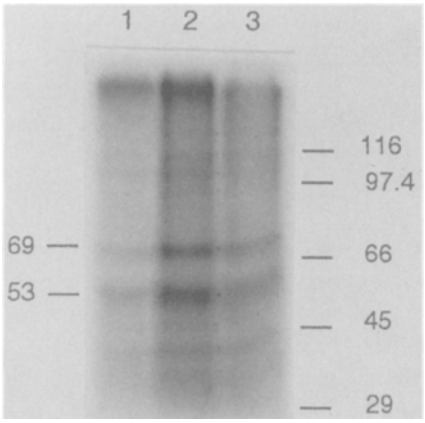


FIG. 5. Phosphorylation of microsomal proteins. Phosphorylation of microsomes was carried out by incubating washed microsomes (20 µg) in 40 µL of 50 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$/1 mM DL-dithiothreitol buffer with ^{32}P-γ-ATP (0.4 mM, 1 Ci/mmol) in the absence (lane 1) and presence (lanes 2 and 3) of 0.5 µg of the purified kinase for 5 min at 37°C. The reaction was stopped by the addition of 20 µL electrophoresis sample buffer (3-fold concentration) and then heated at 95°C for 4 min. A 50 µL aliquot was applied to each lane. Gels were stained, dried, and exposed to Fuji NIF-RX film for three days. Values correspond to molecular weight (kDa) of markers (right) and major phosphopolypeptides (left). Lane 1, control (microsomes alone); lane 2, kinase added; and lane 3, kinase and tyrphostin-25 (0.2 mM) added.

TABLE 2
Reactivation of DGAT by Protein Phosphatase

Addition	DGAT reactivation[a] (% ± SEM)	p^b
None	92 ± 9	—
Okadaic acid (0.1 µM)	88 ± 9	n.s.
NaF (40 mM)	26 ± 4	<0.001
Phosphate (10 mM)	12 ± 15	<0.01
EDTA (1 mM)	6 ± 11	<0.01
Vanadate (2 mM)	45 ± 9	<0.05

[a]Microsomes (1 mg/mL) were pretreated for 10 min at room temperature with cytosol (2.4 mg/mL) and ATP (0.5 mM) in 50 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$/1 mM DTT buffer. Aliquots of treated microsomes (10–15 µg) were incubated for 10 min at 37°C in the same buffer with and without a crude preparation of liver protein phosphatase (0.7 mg/mL) in the presence of the indicated inhibitors and 20 µM of carried over ATP, and then assayed for DGAT activity. DGAT reactivation in the presence of each inhibitor was estimated from the relative increase in DGAT activity of pretreated microsomes caused by incubation with the phosphatase. DTT, DL-dithiothreitol; see Table 1 for other abbreviation.

[b]From a paired *t*-test using the results without inhibitor (92 ± 9%) as reference; n.s., not significant.

the regions of 40 and 100 kDa. The addition of our purified kinase to the incubation medium (lane 2) resulted in the increased phosphorylation of all bands, and this effect was largely prevented when the inhibitor tyrphostin-25 was present in the incubation (lane 3). The fact that the addition of the kinase and the inhibitor did not change the pattern of phosphorylated bands in the microsomes suggested that the endogenous kinase activity remaining in our microsomal preparation was largely the tyrosine kinase which was demonstrated to be present in our purified fraction. By contrast, when the purified kinase was incubated with ^{32}P-γ-ATP and run on SDS–PAGE followed by autoradiography (not shown), no phosphorylated bands were apparent, suggesting that the enzyme does not autophosphorylate.

Reversion of protein phosphorylation and DGAT inactivation with a crude protein phosphatase preparation. We have previously reported that cytosol/ATP dependent inactivation of microsomal GPAT (17) and DGAT (18) activities could be reversed by incubating the inactivated microsomes with a partially purified protein phosphatase from rat liver in the absence of ATP. The reactivation of DGAT from ATP/cytosol-treated microsomes by the phosphatase preparation was now found to be Mg^{++} dependent and could be inhibited by phosphate, NaF, and vanadate, but not by the protein phosphatase inhibitor okadaic acid (Table 2).

Reversibility of microsome phosphorylation by the liver phosphatase preparation was investigated by pretreatment of washed microsomes with the purified kinase and ATP, which resulted in phosphorylation of the microsomes and a 46% decrease in DGAT activity (Fig. 6). Upon removal of the ATP by filtration, incubation of the treated microsomes with the crude protein phosphatase preparation restored DGAT activity while removing most of the microsomes bound ^{32}P (Fig. 6).

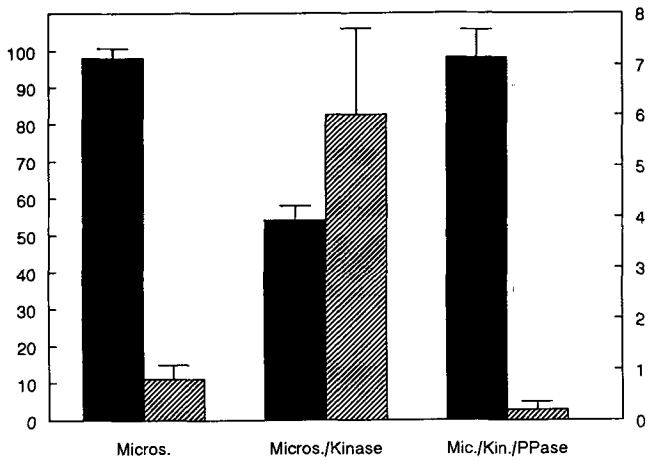


FIG. 6. Reversibility of DGAT inactivation and microsomal phosphorylation. Microsomes (0.3 mg/mL) were incubated at room temperature for 5 min in 50 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$/1 mM DL-dithiothreitol buffer with the purified kinase (2.4 µg/mL) and (0.5 mM) ATP followed by filtration through an Amicon P30 membrane to remove the ATP. The microsomes retained on the filter were resuspended in 0.1 mL of TMD buffer containing 100 µg of the crude phosphatase from liver and incubated for 10 min. Aliquots of this incubation mixture, as well as of the untreated microsomes and the kinase/ATP treated microsomes, were assayed for DGAT activity. Alternatively, ATP was substituted by ^{32}P-γ-ATP (40 Ci/mol) and aliquots corresponding to the untreated, the kinase-treated, and the kinase/phosphatase-treated microsomes were removed for determination of the amount of [^{32}P]phosphate bound to microsomes. Controls were run in parallel in which the kinase and the phosphatase were omitted from the incubations. Results are expressed as percentages of these controls ± SEM (n = 4). Solid bar, DGAT (% control); ruled bar, microsomal [^{32}P]phosphate (nmole Pi/mg). Abbreviation as in Figure 1.

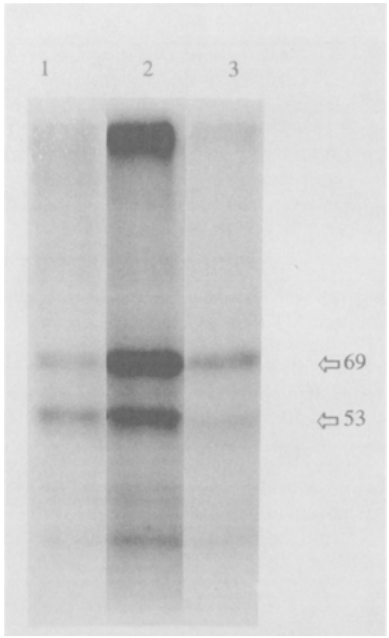


FIG. 7. Phosphatase treatment of phosphorylated microsomal proteins. Washed microsomes were phosphorylated with ^{32}P-γ-ATP in the presence (lanes 2 and 3) and absence (lane 1) of the purified kinase as described in Figure 5. For lane 3, the kinase treated microsomes were filtered on an Amicom P30 membrane, resuspended in TMD buffer, and incubated with crude phosphatase (1 mg/mL) as described in Figure 6. Reactions were stopped by adding electrophoresis sample buffer, and aliquots (20 μg microsomal protein) from the incubation mixtures were applied to the gel. Following electrophoresis, the gel was stained, dried, and exposed to Fuji NIF-RX film for five days. Molecular weight values (kDa) of the major phosphoproteins labelled are indicated. Lane 1, microsomes alone; lane 2, kinase-treated microsomes; and lane 3, kinase/phosphatase-treated microsomes.

Figure 7 shows the dephosphorylation of phosphoproteins from the kinase/ATP-treated microsomes. Treatment with the kinase (lane 2) stimulated the level of ^{32}P-phosphate incorporation into microsomal polypeptides as in Figure 5. Treatment with the liver phosphatase (lane 3) reversed the level of phosphorylation close to control levels (lane 1).

We have observed that if both the purified kinase and liver phosphatase were present in excess with microsomes in the presence of 1 mM ATP, the kinase was able to overcome the phosphatase resulting in net inactivation of DGAT and microsomal phosphorylation (4.5 nmol phosphate/mg microsomes), although not to the level achieved by the purified kinase alone (6.0 nmol phosphate/mg microsomes).

DISCUSSION

The present results confirm our previous findings of an ATP-dependent activity present in the cytosol of adipose tissue, which reduces both microsomal GPAT and DGAT activities *in vitro* (17,18). We have now partially purified that activity. Since we could not measure rates of DGAT inactivation, we were unable to determine the degree of purification of the activity. According to protein recovery in the final pool, the degree of purification attained is on the order of 1000-fold. Considering that this is a regulatory enzyme, higher purification is probably required in order to achieve homogeneity. The purified activity ran at about 67 kDa on a gel filtration column, and showed a single 68 kDa polypeptide band on SDS–PAGE, suggestive of a monomeric enzyme. However, it is possible that a minor component of our purified fraction, not even visible on the SDS gel of Figure 3 but containing the activity, could run at 67 kDa on gel filtration due to interactions with either the gel or with other proteins.

Our results suggest that the activity responsible for DGAT inactivation in the presence of ATP is a protein kinase. This hypothesis is supported by the following observations: (i) DGAT inactivation did not occur when ATP was substituted by β,γ-methylene-ATP (18), (ii) the kinase activity expressed as the phosphorylation of a synthetic peptide was associated with the DGAT inactivating activity through the purification steps, (iii) when endogenous microsomal activity responsible for DGAT inactivation was removed by appropriate washing of microsomes, the microsomal phosphorylating activity was concomitantly lost, (iv) both activities required Mg^{2+} and were inhibited by EDTA, (v) both activities were sensitive to the tyrosine kinase inhibitors genistein and tyrphostin-25, and (vi) both activities could be reversed by treatment with a crude preparation of protein phosphatase from rat liver. In addition, the putative DGAT kinase appeared to be a tyrosine kinase, both because of its sensitivity to established tyrosine kinase inhibitors (genistein and tyrphostin-25) and because of its ability to phosphorylate a synthetic peptide which is used as a general artificial substrate of protein tyrosine kinases. Moreover, the specific activity of the tyrosine kinase increased with the sequential purification steps of the DGAT inactivating activity.

Although a cytosolic enzyme, our protein kinase was associated strongly with the microsomal membranes. Two microsomal polypeptides of 53 and 69 kDa seemed to be substrates of our protein kinase. At this point we cannot assign any of the phosphorylated bands either to the DGAT or GPAT enzymes. However, studies by radiation inactivation of rat liver DGAT suggest that a unit of 68–76 kDa comprising one or more polypeptides is required for activity (25). More recently, rat liver DGAT has been purified to near homogeneity and shown to comprise at least a 60 kDa polypeptide (26).

Furthermore, it has been suggested that a 54 kDa microsomal polypeptide, which is labelled by radioactive iodoacetate but is not phosphorylated by PKA, is a component of adipose GPAT (15). This band may correspond to one microsomal substrate (53 kDa) of our kinase. However, *Escherichia coli* GPAT comprises a single polypeptide of 83 kDa (27) and murine mitochondrial GPAT has been recently identified as a 90 kDa polypeptide (28).

Dephosphorylation of both phosphorylated polypeptides by treatment with a crude protein phosphatase preparation from rat liver was concomitant with the reactivation of DGAT and GPAT activities from microsomes treated with our puri-

fied kinase and ATP, suggesting the involvement of those polypeptides in the said microsomal activities.

Since our previous results indicated that the DGAT enzyme in our microsomal preparations is already in a partially inactivated form (18), the combined effect of kinase inactivation and phosphatase activation should result in a change of rates of triacylglycerol synthesis much higher than the 30-40% change in DGAT and GPAT activities observed in our *in vitro* experiments.

One of the effects of insulin is an increase in adipocyte TAG synthesis, which is not secondary to its effects on lipoprotein lipase, glucose uptake, or fatty acid synthesis (4). The implication of a protein tyrosine kinase in the regulation of two key triglyceride synthetic enzymes, GPAT and DGAT, suggests that in concert with a tyrosyl phosphatase, the kinase could regulate this metabolic pathway. Insulin stimulation of adipose TAG synthesis could be mediated by activation of the phosphotyrosyl phosphatase and inactivation of the putative DGAT/GPAT kinase.

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The Influence of a Fish Oil-Enriched Diet on the Phospholipid Fatty Acid Turnover in the Rabbit Red Cell Membrane *in vivo*

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ABSTRACT: The influence of the polyunsaturated fatty acid (PUFA) composition of the diet on the rate of fatty acid turnover of individual phospholipids in the erythrocyte membrane *in vivo* was studied. Following modification of the fatty acid composition of the membrane phospholipids by the use of a fish oil or a linoleic acid enriched diet, phospholipids—labelled in the unsaturated fatty acid at the 2-position of the glycerol moiety—were introduced into the membrane of freshly isolated rabbit erythrocytes. Thereafter, the labelled erythrocytes were reinjected into the bloodstream of the animal. It appears that, with the exception of 1-palmitoyl,2-linoleoyl phosphatidylcholine, all other phosphatidylcholines disappear faster from the erythrocytes of fish oil-fed rabbits than from the red cells of linoleic acid-fed rabbits. Another parameter, which possibly influences the turnover rates of PUFA containing phospholipids, can be peroxidation. An attempt was made to measure peroxidative damage of lipids *in vivo* by the introduction of 1-palmitoyl,2-*cis*-parinaroyl phosphatidylcholine (PnPC)—a probe to measure oxidative stress—into the membrane of freshly isolated erythrocytes, in the same way as is described for the radioactive phospholipids. The data demonstrate that the fluorescent signal from the PnPC decreases at a fast rate which is independent of the dietary conditions.

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In the last decennia, diets rich in polyunsaturated fatty acids [PUFA, mainly linoleic acid ($C_{18:2n-6}$)] have been recommended for treatment and prevention of atherosclerosis and heart disease, because n-6 PUFA are claimed to have plasma cholesterol-lowering capacities (1–3). More recently n-3 PUFA [eicosapentaenoic acid ($C_{20:5n-3}$) and docosahexaenoic acid ($C_{22:6n-3}$)], present in fish oil, have been recognized as being even more potent than linoleic acid (1,4); n-3 PUFA have a marked triglyceride lowering effect (1,3,5).

However, the beneficial effects of a fish oil-enriched diet seem to be in contradiction with the fact that, as a result of the increased PUFA intake, membranes may accumulate relatively high amounts of PUFA-containing phospholipids (with more than three double bonds) and become more vulnerable to peroxidative damage (6,7). Lipid peroxidation may result in membrane damage and has been correlated with disorders occurring during aging and several diseases including cancer, coronary heart disease, and atherosclerosis (7–12).

Both the composition of the diet, as well as peroxidative damage, can modify the fatty acid composition of membranes. It is not known, however, to what extent the composition of the diet or peroxidative damage will influence the rate of phospholipid fatty acid renewal in the erythrocyte membrane *in vivo*. We have tried to clarify these questions by using a method which enables us to measure the *in vivo* turnover of individual phospholipid species in the erythrocyte membrane (13,14).

First the fatty acid composition of membrane phospholipids is modified *in vivo* by the use of different diets. A fish oil-enriched diet has been compared with a linoleic acid-enriched diet. Then, using a phospholipid transfer protein, trace amounts of radiolabelled and well-defined phospholipids were introduced into the membrane of freshly isolated rabbit erythrocytes. After reinjection of those labelled cells into the animal, the *in vivo* decay of the radiolabel was followed by analyzing the erythrocytes in blood samples that were taken at timed intervals.

Recently, a new method has been described which can be used to measure oxidative stress (*in vitro*) in model membrane systems, erythrocyte membranes, and intact erythrocytes with a naturally occurring fluorescent vegetable fatty acid, parinaric acid [PnA, 9,11,13,15-octadecatetraenoic acid ($C_{18:4}$), 15,16]. The basis for this fluorescent peroxidation assay is provided by the special structure of the PnA molecule, its conjugated double bond system giving rise to the fluorescent properties as well as susceptibility to peroxidation. PnA and parinaric acid containing phosphatidylcholine (PnPC) can be

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Abbreviations: ACD, acid–citrate–dextrose; BHT, butylhydroxy toluene; nsL–TP, nonspecific lipid transfer protein; PC, phosphatidylcholine; 16:0,18:1PC, 1-palmitoyl,2-oleoyl-*sn*-glycero-3PC; 16:0,18:2PC, 1-palmitoyl,2-linoleoyl-*sn*-glycero-3PC; 18:0,20:4PC, 1-stearoyl,2-arachidonoyl-*sn*-glycero-3PC; PC–TP, phosphatidylcholine specific transfer protein; PE, phosphatidylethanolamine; 16:0,20:4PE, 1-palmitoyl,2-arachidonoyl-*sn*-glycero-3PE; PnA, parinaric acid; PnPC, 1-palmitoyl,2-*cis*-parinaroyl PC; PUFA, polyunsaturated fatty acid(s); α-TE, R,R,R-α-tocopherol.

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used as probe molecules to detect lipid peroxidation because peroxidation and decrease in fluorescence are coupled. Applying small amounts of this probe in the abovementioned method can be expected to give information about the occurrence of lipid peroxidation and its influence on phospholipid fatty acid turnover *in vivo*.

Finally, the ability of α-tocopherol to influence lipid peroxidation and phospholipid fatty acid renewal, in particular the protection of PUFA, is examined by varying the vitamin E concentrations in the various diets and applying both approaches described above.

MATERIALS AND METHODS

1-Palmitoyl,2-[1-^{14}C]-oleoyl-*sn*-glycero-3-phosphocholine ([^{14}C]16:0,18:1PC), 1-palmitoyl,2-[1-^{14}C]-linoleoyl-*sn*-glycero-3-phosphocholine ([^{14}C]16:0,18:2PC), and 1-stearoyl,2-[5,6,8,9,11,12,14,15-^{3}H]-arachidonoyl-*sn*-glycero-3-phosphocholine ([^{3}H]18:0,20:4PC) were purchased from Amersham (Buckinghamshire, United Kingdom). 1-Palmitoyl,2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphoethanolamine ([^{14}C]16:0,20:4PE) was obtained from NEN (Du Pont de Nemours, Bad Homburg, Germany).

Corresponding nonradioactive phospholipids, 1-palmitoyl,2-*cis*-parinaroyl phosphatidylcholine (PnPC), 1-palmitoyl,2-oleoyl-*sn*-glycero-3-phosphocholine (16:0,18:1PC), and 1-stearoyl,2-arachidonoyl-*sn*-glycero-3-phosphocholine (18:0,20:4PC) were obtained from Avanti Polar Lipids (Alabaster, Alabama). Egg-yolk PC, cholesterol, and phosphatidic acid were obtained from Sigma (St. Louis, MO). All other chemicals and solvents used were of analytical grade or LiChrosolve grade.

Animals. Thirty-two female Dutch SPF rabbits (HsdPOC.Dutch, 10–11 weeks old) were purchased from Harlan Porcellus (Blackthorn, United Kingdom). They were fed a linoleic- or fish oil (menhaden oil; Oil Milling Division, Rotterdam, The Netherlands) enriched semisynthetic diet (25% Hope Farms RG grains + 75% semisynthetic diet; Hope Farms RG, Woerden, The Netherlands) containing 40 energy% fat. Animals were adapted to the diet by starting with Hope Farms RG and changing the diet every two weeks by replacing 25% Hope Farms with 25% semisynthetic diet. The basal diet contained in g/1000 kJ: calcium caseinate, 14.6; mineral mix, 5.5; vitamin mix, 0.5; fat, 10.8; maizena, 27.1; sawdust, 14.3 and grassflour, 1.4. Two groups received a linoleic acid-enriched semisynthetic diet with different amounts of vitamin E: 187 (group I) or 300 (group II) α-TE/kg diet (1 TE = 1 mg R,R,R-α-tocopherol acetate). Group III and IV received a fish oil-enriched semisynthetic diet with respectively 187 or 300 α-TE/kg diet. The fat content in Group I and II was in energy% 11.4 sunflower seed oil high in linoleic acid, 8.3 sunflower seed oil high in oleic acid, 8 cocoa butter, and 12.3 hydrogenated coconut oil. In Group III and IV, the fat contained (in energy%) 19 fish oil, 3 sunflower seed oil high in linoleic acid, 6.5 sunflower seed oil high in oleic acid, 1 cocoa butter, and 10.5 hydrogenated coconut oil. Diets were prepared in large quantities, subsequently divided in small aliquots and stored frozen at –20°C. The animals were given fresh diets every day. All oils were analyzed for oxidation stability prior to diet preparation and peroxide values were less than 0.1%. The fatty acid composition of the semisynthetic diets is shown in Table 1. The animals were fed the semisynthetic diet for at least four weeks.

Experimental outline. Before starting the *in vivo* experiments, small amounts of blood were taken from the animals for the analyses of the fatty acid and phospholipid compositions as well as the vitamin E contents of serum and erythrocytes. Thereafter, phospholipid turnover was studied as follows. From an animal, a small amount of blood was taken and the erythrocytes were isolated, washed, and incubated with phospholipid vesicles and phospholipid transfer protein as described below. After loading the erythrocyte membrane this way with a radioactive or fluorescent phospholipid, the cells were collected, washed to remove the excess of lipids and protein, and reinjected into the same animal. Thereafter, small blood samples were taken at timed intervals during the next 7 d and analyzed for residual radioactivity or fluorescence present in the erythrocytes.

Preparation of erythrocytes for lipid and vitamin E analysis. Venous blood (7 mL) was obtained from rabbits, using acid–citrate–dextrose (ACD) as anticoagulant. Erythrocytes were pelleted by centrifugation for 10 min at 750 × *g* and washed three times with 4 mL 155 mM NaCl. The buffy coat was carefully removed by aspiration after each wash. After

TABLE 1
Fatty Acid Composition of the Diet[a]

Fatty acid	Group I/II	Group III/IV
<16:0	24.3	25.6
16:0	10.8	13.8
16:1	0.0	5.0
16:2	0.0	1.5
17:0	0.0	1.0
18:0	12.5	5.8
18:1	28.3	21.6
18:2	22.8	7.8
18:3	0.3	0.0
18:4	0.0	1.5
20:0	0.5	0.3
20:1	0.0	1.5
20:2	0.0	0.8
20:5	0.0	7.0
21:0	0.0	0.5
22:0	0.5	1.3
22:1	0.0	0.5
22:5/22:6	0.0	4.5
Σ SAFA	48.6	48.0
Σ MUFA	28.3	28.6
:2	0.0	2.5
:2 (n-6)	22.8	7.8
:3	0.3	0.0
>:3 (n-3)	0.0	13.1

[a]Data represent the major fatty acids as present in the linoleic acid- (Group I and II) and fish oil-enriched (Group III and IV) diets. SAFA: saturated fatty acids; MUFA: monounsaturated fatty acids; :2, :3, >:3: polyunsaturated fatty acids with 2, 3, and more than 3 double bonds, respectively.

the last wash and resuspension in 155 mM NaCl, the haematocrit of the erythrocyte suspension was determined.

Lipid analysis. Erythrocyte samples were extracted according to a modification of the protocol of Rose and Oklander (17). In short, 400 µL packed cells were lysed by adding 400 µL 1 mM EDTA while vortexing. After 15 min at room temperature under nitrogen, 3.2 mL isopropanol containing 0.1 mM butylhydroxy toluene (BHT; Sigma) was added while vortexing. The suspension was vortexed for 90 s under nitrogen. After addition of 2.0 mL dichloromethane (again while vortex-mixing), vortexing was again continued for 90 s. Following centrifugation for 5 min at 1000 × *g*, the supernatant was kept at –20°C under nitrogen until analysis.

Lipids were extracted from 1 mL serum with 4.0 mL isopropanol containing 0.1 mM BHT and 2.5 mL dichloromethane using the above described procedure.

Fluorescence measurements were carried out by transferring 2 mL of the lipid extracts to a thermostated cuvette (T = 25°C) equipped with a magnetic stirring device and using a SLM Aminco SPF 500 Spectrofluorometer (SLM Instruments, Inc., Urbana, IL). The emission wavelength was set at 413 nm with slit width 20 nm; excitation was scanned from 260–360 nm with slit width 2 nm.

For radioactivity measurements, part of the lipid extracts were transferred to scintillation vials and, after evaporation of the organic solvent, radioactivity was determined in a Packard Tri-Carb 1500 Liquid Scintillation Analyzer using Emulsifier Scintillator 299 (Packard, Meriden, CT).

To determine the fatty acid composition of the red cells and serum, total phospholipid extracts were dried under nitrogen, and fatty acid methyl esters were prepared using BF_3 as described previously (18). Analyses were performed on a Perkin Elmer 8500 gas chromatograph (Buckinghamshire, United Kingdom) equipped with either a CP Sil 88, or CP-Wax 52 CB capillary column (both Chrompack, Middelburg, The Netherlands).

To determine the fatty acid composition of the individual phospholipids, extracts of about 500 µL packed cells or 1 mL serum were dried under nitrogen and redissolved in 100 µL chloroform/methanol (1:2, vol/vol). Phospholipids were separated by two-dimensional thin-layer chromatography according to the protocol of Broeckhuyse (19), on thin-layer plates covered with silica 60 (Merck, Darmstadt, Germany). Lipid spots were visualized by fluorescein and scraped off under nitrogen. Phospholipids were extracted from the silica using the method of Bligh and Dyer (20) and subsequently used for analysis of the fatty acid composition.

Vitamin E analysis. To 2 mL of a 20–50% haematocrit cell suspension, 4 mL of ethanol containing 1% vitamin C (Merck), and 0.01% pyrogallol (May and Baker) were added as antioxidants, while vortexing. The apolar fractions were extracted under vortexing with 3 mL of hexane, containing 2.0 µg/mL α-tocopherylacetate (Sigma) as internal standard and 4.4 µg/mL BHT as antioxidant. Thereafter, the mixture was extracted once again with 3.0 mL of hexane. The hexane extracts were stored under nitrogen in the dark at –20°C until analysis.

To determine the vitamin E content of the erythrocyte membrane, extracts were dried under nitrogen and redissolved in 125 µL methanol; 100 µL was injected into the high-performance liquid chromatograph (Gilson, Villiers-le-Bois, France). Vitamin E analysis was performed on an Ultrasphere ODS reversed-phase column (Beckman Instruments, Inc., San Ramon, CA) (250 × 4.6 mm i.d.) and ultraviolet detection (287 nm). Methanol was used as effluent at 1.0 mL/min.

Preparation of erythrocytes for incorporation of phospholipids. From rabbits, adapted to the semisynthetic diet, blood was collected from the ear vein (8 mL/kg bodyweight) in ACD. Erythrocytes were isolated by centrifugation for 10 min at 1000 × *g* and washed two times with 20 mL phosphate buffered saline containing 150 mM NaCl, 5.4 mM Na_2HPO_4, 1.3 mM KH_2PO_4, and 1 mM EDTA (pH 7.4). For phosphatidylethanolamine (PE) incorporation, the cells were washed with "sucrose buffer," containing 280 mM sucrose, 10 mM NaCl, 20 mM glucose, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.4). The buffy coat was carefully removed after each wash.

Preparation of donor vesicles. Vesicles were prepared from two different mixtures of lipids. In a number of experiments, various unlabelled synthetic PC species were used, mixed with 40 mol% of cholesterol, 5 mol% of PA, and trace amounts of [^{14}C]16:0,18:1PC and [^{3}H]18:0,20:4PC, both at a final concentration of 1.0 nCi/nmol phospholipid. The PC-fraction consisted of PnPC, 16:0,18:1PC, and 18:0,20:4PC in a molar ratio of 1:0.25:0.25. The mixture also contained 6 mol% BHT to prevent oxidation.

Alternatively, vesicles were prepared from egg-yolk PC, mixed with an equimolar amount of cholesterol, 6 mol% of PA, and trace amounts of [^{14}C]16:0,18:2PC at a final concentration of 1.1 nCi/nmol PC. The above lipid mixtures were dried under nitrogen, resolved in ethanol (5 µmol PC/mL), and kept under nitrogen at –20°C until further use within one week. Vesicles were prepared in phosphate-buffered saline using the ethanol injection method as described by Batzri and Korn (21).

For studies involving the introduction of labelled PE into the cells, a different method of vesicle preparation was used. Egg-yolk PC was mixed with 40 mol% of cholesterol, 5 mol% of PA, 2.5 mol% BHT, and trace amounts of [^{3}H]18:0,20:4PC and [^{14}C]16:0,20:4PE, both at a final concentration of 0.75 nCi/nmol phospholipid. The lipid mixture was dried under nitrogen and dispersed in sucrose buffer (nitrogen saturated) at a final concentration of 1 µmol phospholipid/mL and vortexed in the presence of a few glass beads. The lipid dispersion was subsequently sonicated under nitrogen for two min, using a Branson (Danbury, CT) sonifier set at 50 W. Vesicle suspensions were stored at 4°C under nitrogen until used (within a few days).

Preparation of transfer proteins. The PC specific transfer protein (PC–TP) was purified from bovine liver (22) and stored at a concentration of 700 µg/mL in 50% glycerol at –20°C. The nonspecific lipid transfer protein (nsL–TP) was also purified from bovine liver (23) and stored at a concentration of 800 µg/mL at –20°C in 50% glycerol. Before use, glycerol was removed by overnight dialysis of the protein so-

lution against a 100-fold volume of phosphate-buffered saline or sucrose buffer (in the case of nsL–TP) at 4°C. The PC–TP solution was used as such, but the nsL–TP protein solution was first concentrated against flake polyethylene glycol (Aquacide III; Calbiochem, La Jolla, CA).

Incorporation of labelled phospholipids into erythrocytes. A 30% (vol/vol) suspension of cells was incubated at 37°C during 60 min (in closed tubes) in a slowly shaking waterbath in the presence of labelled donor vesicles and 2.5 μM PC–TP in phosphate-buffered saline. The ratio of erythrocyte phospholipid to vesicle phospholipid was 50 (the ethanol concentration was less than 0.5%). Cells were isolated by centrifugation at 1000 × *g* for 10 min, and the residual vesicles and PC–TP were removed by one additional wash with an excess of 155 mM sterile NaCl.

For the incorporation of radiolabelled PE, erythrocytes were incubated at 37°C during 60 min in a slowly shaking waterbath with nsL–TP (1.2 mg/mL packed cells) and radiolabelled vesicles. The ratio of erythrocyte phospholipid to vesicle phospholipid was 20. After incubation, the erythrocyte suspension was diluted twice with sucrose buffer, and the cells were subsequently isolated by centrifugation at 1000 × *g* during 10 min. The residual vesicles and nsL–TP were removed by two additional washes with an excess of 155 mM sterile NaCl.

In vivo *fatty acid renewal of various phospholipid species and lipid peroxidation.* The labelled erythrocytes were resuspended in 155 mM sterile NaCl and reinjected into the ear vein of the rabbit. After one hour, and thereafter at timed intervals during one week, 3 mL blood samples were taken. Lipids were extracted from erythrocytes, and the residual radioactivity or fluorescence was determined following the above described procedures. The measured radioactivity or fluorescence is plotted semilogarithmically against time and the half-time values of the various PC-species and PnPC are calculated using the formula:

$$y(t) = A \cdot e^{-\delta t} \quad [1]$$

in which $y(t)$ = the amount of label at time t, A is a constant factor and δ = rate of loss of radioactivity or fluorescence.

RESULTS

Fatty acid composition of serum and serum PC. The fatty acid composition of serum for the different dietary groups is given in Table 2. The fish oil-enriched diet causes drastic changes in the fatty acid pattern of serum of rabbits, compared to the linoleic acid-enriched diet, characterized by large increases in n-3 PUFA, eicosapentaenoic acid ($C_{20:5}$), docosapentaenoic acid ($C_{22:5}$), and docosahexaenoic acid ($C_{22:6}$), in combination with decreased $C_{18:2n-6}$ concentrations. The use of different diets results also in differences in fatty acid composition of serum PC (Table 2).

Fatty acid composition of total phospholipids and the individual phospholipid classes PC and PE in the erythrocyte membrane. The fatty acid composition of the erythrocyte membrane (Fig. 1) of rabbits fed a linoleic acid-rich diet is similar to that of serum (Table 2). The rabbit erythrocyte membrane contains $C_{18:2n-6}$ (35%) as major fatty acid, and this amount is somewhat lower than that of serum. Feeding fish oil to rabbits resulted in drastic changes in the fatty acid pattern of their red cell phospholipids. There is a large accumulation of n-3 PUFA, $C_{20:5}$ (9%), $C_{22:5}$ (7%), and $C_{22:6}$ (8%) in combination with lower $C_{18:2n-6}$ concentrations (14%). Surprising is the low amount of $C_{20:5}$ and the high amount of $C_{22:5}$ when compared to the situation in serum. The amount of $C_{20:4n-6}$ is not changed by the use of fish oil. The almost constant level of $C_{20:4n-6}$ in erythrocytes is in agreement with previously published data on rabbits (24), but not with those of monkeys (25).

TABLE 2
Fatty Acid Composition of Serum and Serum PC from Rabbits[a]

	Group I		Group III	
Fatty acid	Serum (%)	Serum PC (%)	Serum (%)	Serum PC (%)
16:0	14.1	16.0	18.1	17.8
18:0	18.8	26.0	14.8	24.4
18:1	23.3	12.8	17.6	11.1
18:2	39.1	41.0	14.6	22.9
20:4	4.8	4.4	5.4	5.9
20:5	—	—	18.5	10.0
22:5	—	—	3.2	2.2
22:6	—	—	8.1	5.9

[a]Rabbits were fed a linoleic acid-rich (Group I) or a fish oil-rich (Group III) semisynthetic diet, both supplemented with 187 α-TE/kg diet, for at least four weeks. Total lipid extracts of serum and serum phosphatidylcholine (PC) were analyzed for their fatty acid composition as described in the Materials and Methods section. Data presented are the averages of values obtained from duplicate analyses of serum and serum PC from two different animals and are expressed as percentage of the total major fatty acids. A dash (—) indicates a value of less than 0.5%.

The total PUFA content, including C18:2n-6, did not change by the diet; it remained 43–44% of the total fatty acid composition of the membrane, while the double bond index (mean number of double bonds per fatty acid) is increased from 1.2 for erythrocytes of linoleic acid-fed rabbits to 2.0 for erythrocytes of fish oil-fed animals. A theoretical lipid peroxidation susceptibility index (6) for the erythrocyte, based on PUFA oxidation susceptibility indices of 1, 3, 4, and 5 for 18:2, 20:4, 20:5/22:5, and 22:6, respectively, is increased from 63 (group I) to 135 (group III). These oxidation related membrane characteristics indicate that the PUFA-enriched erythrocytes are expected to be more susceptible to lipid peroxidation.

The use of fish oil also results in characteristic differences in fatty acid composition of the individual phospholipid classes PC and PE (Fig. 2). In both these classes, 60–70% of the C18:2n-6 is replaced by n-3 PUFA (C20:5, C22:5, and C22:6). Interestingly, the decrease in the relative amount of 18:1 as observed in the total red cell fatty acids in fish oil-fed rabbits (Fig. 1, III), comes to the exclusive account of a change in the fatty acid composition of the PE (Fig. 2B).

In vivo *turnover and lipid peroxidation in the erythrocyte membrane.* The fish oil-enriched diet drastically changed the

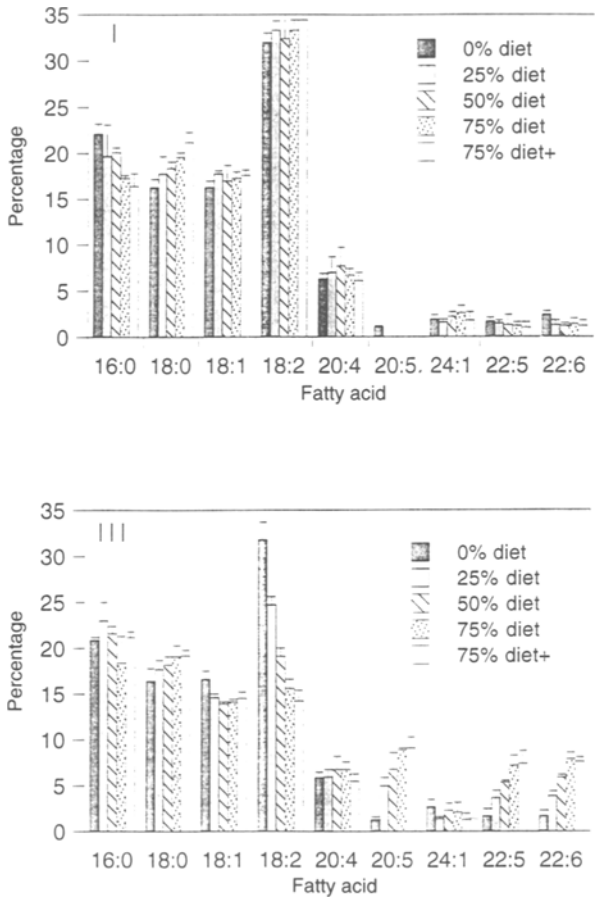


FIG.1. Fatty acid composition of rabbit erythrocyte membranes during adaptation to the diet. Rabbits were fed a linoleic acid-rich (Group I) or a fish oil-rich (Group III) semisynthetic diet both supplemented with 187 α-TE/kg diet. Animals were adapted to the diet by starting with Hope Farms RG (Woerden, The Netherlands) and changing the diet every two weeks by replacing 25% Hope Farms with 25% semisynthetic diet: 25, 50, and 75% semisynthetic diet. Just before the turnover experiments started, an additional analysis was done. Total lipid extracts of erythrocytes were analyzed following the procedures described in the Materials and Methods section and data presented are the average of values obtained from duplicate analyses of erythrocytes from four or eight rabbits. Fatty acids corresponding to less than 0.5% are not indicated.

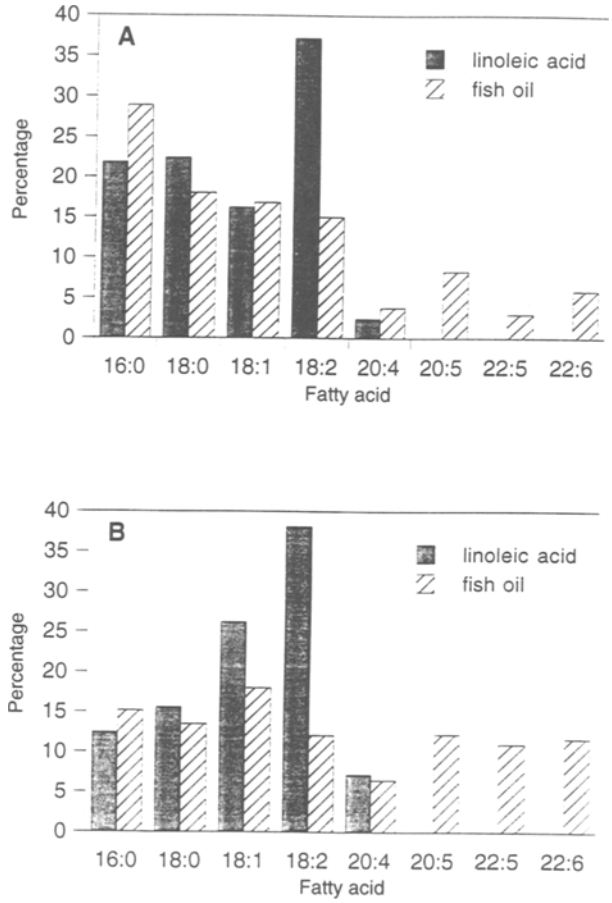


FIG. 2. Fatty acid composition of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in rabbit erythrocyte membranes. Rabbits were fed a linoleic acid-rich or a fish oil-rich semisynthetic diet, both supplemented with 187 α-TE/kg diet for at least four weeks. The individual phospholipids (PC, PE) were separated and analyzed for their fatty acid composition as described in the Materials and Methods section. Data are the average of values obtained from duplicate analyses of PC and PE of erythrocytes from two rabbits. Fatty acids corresponding to less than 0.5% are not indicated. A and B: fatty acid composition of PC and PE, respectively.

fatty acid composition of the rabbit erythrocyte membrane. Those membranes contain high quantities of n-3 PUFA (group III) in contrast to the other group which contains n-6 PUFA (group I). Both types of modified red cells have been used to investigate the influence of the PUFA composition of the diet on the *in vivo* turnover of various phospholipid fatty acids.

Small amounts of radiolabelled phospholipids were introduced into the membrane of freshly isolated erythrocytes in such a way that less than 1% of the native PC or PE in the erythrocyte was replaced by the radiolabelled markers. As documented extensively in previous studies, such a low amount of replacement does not affect the structural and functional characteristics of the erythrocyte membrane nor the overall cellular properties to any measurable extent (13). Erythrocytes, containing the radioactive phospholipids, were reinjected into the animals and at various time points thereafter small samples were taken and analyzed for residual radioactivity present in the total phospholipid extracts. The actual half-time values for the loss of the different phospholipid species from circulation have been calculated using the formula as shown in the Materials and Methods section and are presented in Table 3. As discussed previously (14), the observed half-time values for the disappearance of the label from circulation need to be corrected for the contribution of cell sequestration, in order to obtain information of the actual fate of the probe molecules in the circulating erythrocytes. The average half-time value for red cell survival in rabbits on a linoleic acid-rich diet has been determined to be 12 d (13), but it is not known whether this parameter changes as a consequence of the fish oil-rich diet. However, the observation that the half-time values for the *in vivo* survival of both the

TABLE 3
Half-Time Values of *in vivo* Survival of Various Phospholipid Molecular Species in Rabbit Erythrocyte Membranes[a]

	Half-time values in days							
	Group I		Group II		Group III		Group IV	
Phospholipid	obs	cor	obs	cor	obs	cor	obs	cor
16:0,18:2* PC	1.4	1.5	1.4	1.5	1.4	1.5	1.4	1.5
16:0,18:1* PC	3.7	5.3	3.2	4.3	3.1	4.2	3.2	4.3
18:0,20:4* PC	3.8	5.6	3.4	4.6	3.2	4.3	3.3	4.4
18:0,20:4* PC**	3.6	5.1	n.d.		3.0	4.1	n.d.	
16:0,20:4* PE**	6.6	14.5	n.d.		4.9	8.2	n.d.	
PnPC	1.3	1.4	1.2	1.3	1.4	1.5	1.6	1.7

[a]Rabbits were fed a linoleic acid-rich semisynthetic diet with 187 (Group I) or 300 α-TE/kg diet (Group II) or a fish oil-enriched semisynthetic diet with 187 (Group III) or 300 α-TE/kg diet (Group IV). The observed (obs) and corrected (cor) half-time values on the removal of individual species from the circulating cells are calculated from the formulas as shown in the Materials and Methods section and the Results section, respectively. The asterisk (*) indicates the position of the label. Data marked ** are obtained with radiolabelled phospholipids which were introduced into the erythrocytes with the nonspecific lipid transfer protein, whereas the others are obtained using the PC specific transfer protein. The data are the average of values obtained from analyses of decay of radioactivity and fluorescence, respectively, of erythrocytes from two or three rabbits; each time point is analyzed in duplo; n.d. is not determined; PnPC, 1 palmitoyl, 2-*cis*-parinaroyl PC. See Table 2 for other abbreviations.

TABLE 4
***In vivo* Daily Replacement of Rabbit Erythrocyte Membrane Phospholipids[a]**

	Daily replacement (% of total erythrocyte PC/PE)			
Phospholipid	Group I	Group II	Group III	Group IV
16:0,18:2* PC	24.8	24.0	10.0	9.5
16:0,18:1* PC	3.1	3.8	4.0	3.8
18:0,20:4* PC	0.4	0.5	0.9	0.9
18:0,20:4* PC**	0.5	n.c.	1.0	n.c.
16:0,20:4* PE**	0.5	n.c.	0.8	n.c.

[a]Rabbits were fed a linoleic acid-rich semisynthetic diet with 187 (Group I) or 300 α-TE/kg diet (Group II) or a fish oil-enriched semisynthetic diet with 187 (Group III) or 300 α-TE/kg diet (Group IV). The daily replacement of each species can be estimated on the basis of the turnover data and the abundance of each species (Fig. 2) and are expressed as % of the total phosphatidylcholine (PC) or phosphatidylethanolamine (PE) complement of the erythrocyte membrane (n = 2–3). The asterisk (*) indicates the position of the label. Data marked ** are obtained with radiolabelled phospholipids which were introduced into the erythrocytes with the nonspecific lipid transfer protein, whereas the others are obtained using the PC specific transfer protein; n.c., not calculated.

16:0,18:2PC and PnPC do not, if at all, experience any appreciable changes, strongly indicates that the change in diet does not affect the lifetime of the circulating erythrocytes. Hence, the observed half-time values for the *in vivo* disappearance of radiolabel and fluorescence signal, respectively, can be corrected for the contribution of cell sequestration (Table 3) by using the previously applied formula:

$$y(t) = A \cdot e^{-(\delta - \delta ery)} \quad [2]$$

in which $y(t)$ = amount of radiolabel at time t, A = a constant, δ = rate of loss of radioactivity or fluorescence, and δery = rate of removal of erythrocytes from circulation. The data for the fish oil-fed rabbits (groups III and IV) show that differences do occur in the rates at which the individual species disappear from the circulating cells. With the exception of 16:0,18:2PC, all other phospholipids that had been introduced into the erythrocytes of fish oil-fed rabbits showed a (slight) decrease in the half-time values of their *in vivo* survival.

Taking the half-time values presented in Table 3, the fatty acid analysis data in Figure 2 (for instance, $C_{20:4n-6}$ comprises 2.4% of the total fatty acids of the PC) and making the assumption that the majority of the PUFA occupy the 2-position in the phospholipid molecules, it is possible to estimate the amounts of various individual PC and PE species which are replaced per day. Thus, 18:0,20:4PC represents at most 4.8% of the total PC pool, and using the half-time value for its *in vivo* survival in the linoleic acid fed rabbits of 5.6 d (Table 3), it follows that the removal of 18:0,20:4PC from circulation contributes to 0.4% of the total red cell PC per day only. But still, this is an overestimation because part of $C_{20:4n-6}$ will be present in combination with C16:0 instead of $C_{18:0}$, which means that the relative amount of 16:0,20:4PC will in fact be less than 4.8% of the total PC. Similar estimations for the other species have been made, and the results are shown in Table 4. It is obvious that a somewhat higher PC turnover takes place in the fish oil-fed group, with the exception of the 16:0,18:2 species. This supports the idea that direct exchange of intact PC molecules between serum and erythrocytes plays an important role, because the supply of 16:0,18:2PC from the serum and the amount in the erythrocytes are less in the fish oil group than in the linoleic acid-fed group. The opposite effect is seen for 18:0,20:4PC, which is increased in both serum and erythrocytes of fish oil-fed rabbits and, as a consequence, its contribution to the turnover of the entire PC pool in the erythrocytes increases by about 100%.

Another parameter which possibly influences the turnover rates of phospholipids can be found in the process of lipid peroxidation. To investigate this effect, small amounts of PnPC, a probe to measure oxidative stress, were introduced into the membrane of freshly isolated erythrocytes in the same way as is described for the radioactive phospholipids. Erythrocytes, containing the PnPC, were reinjected into the animals, and at various time points thereafter, small samples were taken and analyzed for residual fluorescence present in the total phospholipid extracts. The half-time value found for PnPC in erythrocytes from fish oil-fed rabbits is 1.4 d and differs not from that found in erythrocytes from linoleic acid-fed rabbits (1.3 d).

Vitamin E levels in the rabbit erythrocyte membrane. Figure 3 gives the results of vitamin E analyses of rabbit erythrocytes as a function of the diet. Although both linoleic- and fish oil-enriched diets contain equal amounts of vitamin E (187 α-TE/kg diet), the α-tocopherol content of rabbit erythrocytes is drastically lowered in fish oil-fed rabbits (group III), be it still somewhat higher than the starting values. Supplementation of the linoleic acid-enriched diet with 300 α-TE/kg diet (group II) increases drastically the vitamin E lev-

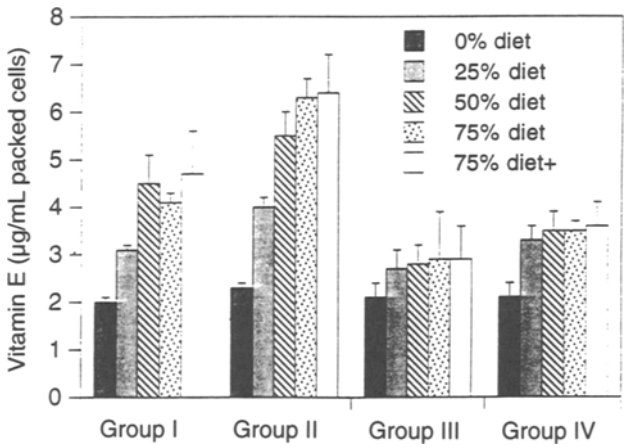


FIG. 3. The effect of the diet on vitamin E levels in the rabbit erythrocyte membrane. Rabbits were fed a linoleic acid-rich semisynthetic diet with 187 (Group I) or 300 α-TE/kg diet (Group II) or a fish oil-rich semisynthetic diet with 187 (Group III) or 300 α-TE/kg diet (Group IV). Animals were adapted to the diet by starting with Hope Farms RG and changing the diet every two weeks by replacing 25% Hope Farms with 25% semisynthetic diet: 25, 50, and 75% semisynthetic diet. Just before the turnover experiments started, an additional analysis was done. Erythrocyte membrane extracts were analyzed for their vitamin E levels as described in the Materials and Methods section. Data presented here are the average of values obtained from duplicate analyses of erythrocytes from four or eight rabbits. Company source as in Figure 1.

els of the rabbit erythrocyte membrane. On the other hand, supplementation of fish oil-enriched diet with extra vitamin E (300 α-TE/kg diet, group IV) has only a small positive effect on those levels in the erythrocyte, and the vitamin E concentration remains still below that in the group fed a linoleic acid-rich diet with 187 α-TE/kg diet.

The serum and erythrocyte lipid fatty acid patterns of the rabbits supplemented with 300 α-TE/kg diet closely resemble the 187 α-TE/kg diet fatty acid patterns (data not shown) and are in agreement with previously published data (24).

The extra addition of vitamin E to the diet has no influence on the turnover values of red cell PC fatty acids in fish oil-fed rabbits and a small turnover enhancing effect on red cell PC fatty acids in linoleic acid-fed rabbits (Table 3). Similarly, it has neither an effect on the phospholipid replacement per day (Table 4), nor on the half-time value of the disappearance of PnPC fluorescence (Table 3).

DISCUSSION

The data presented here demonstrate that the fish oil-enriched diet causes profound changes in the phospholipid fatty acid pattern of serum and erythrocyte membranes of rabbits. Feeding fish oil to rabbits results in a high amount of n-3 PUFA ($C_{20:5}$, $C_{22:5}$, and $C_{22:6}$) in the phospholipids of both serum and red cell membranes, and these data are in agreement with previously published results on rabbits (24,25), monkeys (26), rats (27,28), and humans (29,30). It is not known, however, to what extent the composition of the diet will influence the rate of fatty acid renewal of individual phospholipids in the erythrocyte membrane *in vivo*. To study this in more detail, we introduced phospholipids, labelled in the unsaturated fatty acid at the 2-position, in the rabbit erythrocyte membrane as described recently (14). Only trace amounts of PC and/or PE are introduced into the red cell membranes which will be diluted in the regular PC/PE pools (13,31) *via* transbilayer redistribution (32–34). After reinjection of the labelled cells into the rabbit, decay of radioactivity was measured in time, and the renewal rate of the phospholipid fatty acids in the red cell membrane was calculated. It appears that the fatty acid composition of the diet affects the rate of renewal of individual phospholipids. The labels from 16:0,18:1PC, 18:0,20:4PC, and 16:0,20:4PE disappear faster from the erythrocytes of fish oil-fed rabbits than from the red cells of linoleic acid-fed rabbits (Table 3). An exception is observed for the disappearance of the radiolabel from 16:0,18:2PC. The half-time value of this species did not change by the use of a fish oil-enriched diet. This observation might be explained by the fact that the rate of renewal of this phospholipid is already extremely high (half-time value is 1.5 d), and therefore small differences are not noticeable anymore.

Another way of looking at the influence that the composition of the diet may have on the renewal of the phospholipids in the red cell is by calculation of the replacement of these phospholipids in the erythrocyte membrane per day (Table 4). It shows that in erythrocytes of fish oil fed rabbits an increase in replacement of 18:0,20:4PC by about 100% per day is found compared to erythrocytes of linoleic acid-fed rabbits. If we consider the fact that in the serum of fish oil-fed rabbits more PC with arachidonic acid at the 2-position is present, and assuming that the total phospholipid content remains constant throughout (5), the conclusion might be drawn that the replacement of individual PC species depends on the amount of these species in the serum. The observation that the fatty acid composition of the PC present in the serum resembles that of the erythrocytes, to a large extent, suggests that direct exchange of intact PC molecules between erythrocyte and serum may play a role. The data on 16:0,18:2PC support this conclusion, because the concentration of this PC is lower in serum and erythrocytes of fish oil-fed rabbits, thus resulting in a lower contribution of this species to the daily exchange of the entire PC pool. On the other hand, loss of radioactivity from the reporter molecules can also be due to a process of de-/reacylation, since the radiolabel was present in the fatty acid residue at the 2-position of the phospholipids and unlabelled fatty acid, free, or esterified to other complex lipids is present in excess. However, previous results (14) suggest that the loss of molecular species of PC from the circulating red cell is due to the exchange of intact PC molecules to the serum.

Due to the preferred localization of PE in the inner membrane layer of the rabbit erythrocyte [18:82, out/in (13,31)], exchange of erythrocyte PE with serum PE might play a minor role. Therefore, the observed higher turnover of 16:0,20:4PE in fish oil-fed rabbits (Table 3) could be due to an increase in metabolic turnover (35,36).

Whatever mechanism of renewal is operative, the total amounts of both phospholipids in the red cell membrane remain fairly constant throughout (5,29). This implies that the loss of a phospholipid from the erythrocyte membrane has to be compensated for by the uptake of a molecule with an identical polar headgroup from the serum pool or by uptake of a lysophospholipid from the serum followed by reacylation with fatty acid at the inner membrane layer of the red cell, as suggested by the experimental evidence presented by Renooij *et al.* (35–38).

Another factor which may influence the turnover of PUFA containing phospholipids can be peroxidation. As a result of an increased PUFA intake due to the fish oil diet, an accumulation of n-3 PUFA containing phospholipids in the erythrocyte membrane appears, and these red cells become more vulnerable for peroxidative damage.

An attempt was made to measure peroxidative damage of lipids *in vivo* by using parinaroyl PC [PnPC(n-3)]. The data demonstrate that the fluorescent signal from the PnPC decreases at a fast rate (Table 3) which is independent of the dietary conditions. Assuming that this loss of fluorescent signal is not due to exchange of the PnPC with serum PC, because an equivalent phospholipid is not present in the serum, peroxidation may be responsible for the decay. This seems in contradiction with the increase in the peroxidation susceptibility index from 63 for erythrocytes of linoleic acid-fed rabbits to 135 for erythrocytes of fish oil-fed rabbits, since in the latter case a higher half-time value for the disappearance of this probe might have been expected as a consequence of the higher buffering capacity of the high amount of n-3 PUFA to cope with the radicals formed. On the other hand, the fluorescent PnA is very sensitive to peroxidation (39), and the assumption seems justified that it has a higher sensitivity toward peroxidation than the other naturally occurring n-3 fatty acids. One possible explanation could be that in both cases the PnPC will be the first phospholipid to be peroxidized, which is in agreement with *in vitro* results published by van den Berg *et al.* (26).

Finally, the ability of α-tocopherol, the major lipid-soluble chainbreaking antioxidant in the erythrocyte membrane (40), to influence lipid peroxidation and phospholipid fatty acid renewal *in vivo* is examined. Although both diets contain equal amounts of vitamin E (187 α-TE/kg diet), the α-tocopherol content of rabbit erythrocytes was drastically lowered in fish oil-fed animals when compared to linoleic acid-fed rabbits, and these results are in agreement with data from Nair *et al.* (41).

The low amount of vitamin E in the red cell of fish oil-fed rabbits may be a result of the low amount of this antioxidant in the plasma, because (i) vitamin E can exchange between plasma and erythrocytes (42), (ii) its concentration is related to the concentration of plasma lipids (43), and (iii) in humans the ratio between plasma and the red cell vitamin E appears to be constant (approximately 3:1), irrespective of large differences in vitamin E intake (40). Indeed, in plasma of fish oil-fed rabbits, a reduction in α-tocopherol concentration is observed in comparison with linoleic acid-fed rabbits, the levels being 14.0 and 17.8 μg α-tocopherol/mL serum, respectively, although it remains as yet unknown whether this change is due to a reduced lipid content in the plasma of fish oil-fed rabbits. However, this decrease in α-tocopherol content is in agreement with data published for men (41).

On the other hand, this decrease in circulating plasma α-tocopherol in fish oil-fed rabbits could be a reflection of the increased demand for antioxidants as result of the increased PUFA intake. However, in the erythrocytes of either linoleic acid- or fish oil-fed rabbits supplementation with extra α-tocopherol did not result in a slower turnover of phospholipid fatty acids or in a decreased lipid peroxidation. This could indicate that the vitamin E level in the red cells of either rabbit is already sufficiently high, even without extra supplementation. This view is supported by the observation that (i) the erythrocyte phospholipid fatty acid pattern was similar irrespective of feeding rabbits with either 187 or 300 α-TE/kg of vitamin E, which is in agreement with observations in monkey erythrocytes (25) and (ii) the lowest concentration of dietary vitamin E already provides a level of approximately one molecule of vitamin E per 1000 lipid molecules in the red cells (40).

Concluding, it appears that the fatty acid composition of the diet affects the rate of phospholipid fatty acid renewal in the erythrocyte membrane *in vivo*.

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The Effects of Dietary Phospholipids Enriched with Phosphatidylethanolamine on Bile and Red Cell Membrane Lipids in Humans

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ABSTRACT: The role of phospholipids in biliary cholesterol solubilization and crystallization has only recently begun to be appreciated. Phospholipid vesicles are believed to be the metastable carrier from which cholesterol nucleates. Cholesterol crystallization is influenced by the phospholipid species in bile. Feeding rats and hamsters with diets enriched in phospholipids or their precursors, especially ethanolamine, resulted in reduced cholesterol saturation of bile. Although whole phospholipids are normal dietary constituents, the effects and safety of phospholipid components have not been tested in humans. In the present study, we have evaluated the effects of a dietary phospholipid mixture, enriched with phosphatidylethanolamine, on human bile and red blood cell membrane lipid composition. Five ambulatory volunteers having a chronic indwelling T-tube, with an intact enterohepatic circulation, were investigated. Thirty-six grams of phospholipids (54% phosphatidylethanolamine, 54% linoleyl acyl chains) were added to their daily diet for fourteen days. Biliary nucleation time, cholesterol carriers, as well as plasma, red blood cell membrane, and bile lipid compositions, were monitored. Following phospholipid supplementation, the proportion of linoleyl chains (18:2) in biliary phospholipids increased significantly from 31.1 ± 1.2 to 37.7 ± 5.3%, while that of oleyl chains (18:1) decreased from 11.4 ± 1.6 to 9.6 ± 1.1%. These changes were accompanied by an increase of linoleate and its metabolite, arachidonate, in red cell membranes. Phospholipid feeding did not cause any side effects, and no significant changes in biliary nucleation time, cholesterol, phospholipid, or bile salt concentrations, or in the distribution of cholesterol within micelles or vesicles. We conclude that phospholipid feeding is safe, and can be effective as a vehicle for lecithin fatty acyl chain modulation of bile and lipid membranes. These findings may provide a basis for a controlled modulation of biliary phospholipids to increase cholesterol solubility in bile.

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The water-insoluble cholesterol is secreted into bile in association with phospholipids, in the form of vesicles (1,2). Bile salts are secreted separately, and subsequently form (together with phospholipids) various cholesterol solubilizing aggregates. The presence of micelles, phospholipid vesicles, and liquid crystals is widely accepted. The presence of additional lamellar structures is controversial (3,4). Phospholipids are essential components in all these aggregates (except simple micelles). Bile salts were at the forefront of biliary research for over two decades. The role of phospholipids and their various molecular forms has only recently begun to be appreciated.

First it was demonstrated that phospholipid vesicles were the metastable carrier from which cholesterol nucleated, particularly when their cholesterol/phospholipid ratio was high (5,6). Subsequently, it was shown that the addition *in vitro* of minute amounts of phospholipids, particularly those with saturated acyl chains, normalized the nucleation time of lithogenic biles (7). The addition of equivalent amounts of bile salts had no effect on nucleation. It was demonstrated that changes in the head group or fatty acyl chains of phospholipids, without any change in their concentration, had major effects on cholesterol carriers and the nucleation time in model and in human biles (8). The process of cholesterol crystallization is also significantly influenced by the phospholipid species in bile (9,10).

All these results stimulated the interest in the qualitative and quantitative modulation of biliary phospholipids. The phospholipids in bile are composed primarily of a mixture of lecithin molecular species having palmitic or stearic acid in the *sn*-1 position with more unsaturated acyl chains in the *sn*-2 position (11,12). Experimental (13–16), as well as clinical, studies (17,18) demonstrated that biliary phospholipid secretion and composition can be modulated by dietary means. Feeding diets enriched in phospholipids and their components to rats and hamsters revealed that ethanolamine administration markedly reduced cholesterol concentration and the cholesterol saturation index (CSI) of bile (19). The effects and safety of some phospholipid components have not been tested in humans, although whole phospholipids are normal dietary constituents (in eggs, soybean, etc.). In the present study, we have evaluated the effects of a dietary phospholipid mixture,

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Abbreviations: CSI, Cholesterol saturation index; FC, free cholesterol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; RBC, red blood cell.

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enriched with phosphatidylethanolamine (PE), on human bile and red blood cell (RBC) membrane lipid composition.

MATERIALS AND METHODS

Subjects. Six experiments were carried out in five ambulatory volunteers having a chronic indwelling T-tube, with an intact enterohepatic circulation. One subject was studied twice. All patients had undergone cholecystectomy, choledochotomy, and T-tube placement for gallstones—four had cholesterol and one (Patient OA; see Table 1) pigment stones. The study was approved by the local ethical committee, and informed consent was obtained from all participants. The demographic and clinical data are presented in Table 1. All patients were well nourished and their hepatic, renal, and thyroid functions were within normal limits. They had no evidence of obesity (>125% ideal body weight), recent weight loss (>10% during six past months), hypercholesterolemia (cholesterol >250 mg/dl), or diabetes mellitus, nor were they receiving any medication (including oral contraceptives) which could affect the parameters studied.

Study protocol. To ensure an intact enterohepatic circulation, the T-tube had been clamped for a minimum of 10 d before initiating the study. Patients were kept on a constant diet designed to be nutritionally adequate, consisting of 25% protein, 30% fat, 45% carbohydrates, and a total of 1600–2000 kcal per day, from the clamping day and throughout the study period. After ten days on this diet, 36 g of phospholipids (Lecithin granules; Lucas Meyer, Hamburg Germany; for composition see Table 2) were added in three divided doses to this diet for fourteen days. Thereafter, the subjects were switched back to the basic diet for another fourteen days. Bile was collected by gravity from the T-tube for 30 min at predesignated times. In between sampling, the T-tube was kept clamped throughout the study period. Blood and bile samples were obtained after an overnight fast at two-week intervals, each time on two consecutive days. The first pair of samples (sample A) was obtained two days before addition of the lecithin granules to the diet (day 0, day 1), the second (sample B) on the last two days of the lecithin-supplemented diet (day 14, day 15), and the third (sample C) on the last two days of the basic diet alone (day 28, day 29). Blood samples were collected for analysis of plasma lipids, as well as for RBC membrane lipids and fatty acid composition. Bile samples were used for determination of lipids, nucleation time, cholesterol carriers, and fatty acid composition of biliary phospholipids. Stone composition was determined after drying, pulverization, and dissolution in isopropanol as previously described (20).

Bile analysis. An aliquot (1 mL) of the collected hepatic bile was frozen immediately at −70°C for subsequent lipid analysis, performed within four weeks. The remaining bile was ultracentrifuged at 192,000 *g* for one hour at 25°C to remove cell debris. Samples of the supernatant bile were used to determine the nucleation time and the distribution of biliary cholesterol between the vesicular and micellar carriers by gel filtration chromatography as described elsewhere (6,21,22). Bile lipids were extracted by the method of Folch *et al.* (23). Bile acid concentration was determined enzymatically (24), cholesterol was determined by the method of Abbell *et al.* (25), and phospholipids as described by Bartlett (26). The CSI was calculated using the critical tables of Carey (27). Phosphatidylcholine (PC) and PE were separated by chromatography on a column of silicic acid (28). All samples were tested for purity after the separation by thin-layer chromatography (Silica gel 60, 0.25 mm; Merck, Darmstadt, Germany) employing chloroform/methanol/acetic acid/H_2O (100:20:12:4, by vol). Aliquots (5 µL) of the fresh bile incubated at 37°C were examined daily under polarized light. Nucleation time was defined as the time at which at least three cholesterol crystals were observed and found again in the next day's sample (22). The samples were observed up to 30 d. The gross appearance and chemical analysis of the stones, as well as the presence or absence of cholesterol monohydrate crystals in the sediment, were used to classify stone composition (29).

Plasma lipid analysis. Ten mL of venous blood drawn into glass tubes containing EDTA (1 mg/mL of blood) were transferred to the laboratory in iced water for immediate separation by low-speed refrigerated centrifugation (1000 *g*, 4°C) for ten minutes. Sodium azide (1 mg/mL plasma) was added, and the plasma was then kept at 4°C for up to five days prior to determination of total plasma lipids by enzymatic colorimetric testing. Triglycerides were determined with a kit from Sigma Co. (St. Louis, MO), and phospholipids, were determined with total and free cholesterol (FC) by kits from Boehringer Mannheim GmbH (Ingelheim, Germany).

Preparation and chemical analysis of RBC membrane lipid extracts. After plasma separation, the erythrocytes were washed three times with three volumes of a cooled isotonic solution containing 0.15 M NaCl, 10^{-5} M EDTA. The buffy coat was removed by aspiration after each wash. Finally, the

TABLE 1
Patient Characteristics

Subject	Sex	Age (yr)	Weight (kg)	Height (cm)	Postoperative (d)	Procedure
LM	M	56	64	170	30	Portoenterostomy
TN	M	35	62	170	40	Choledocotomy
W	F	76	59	146	45	Choledocotomy
XJ	M	65	87	175	300	Choledocotomy
~~OA~~	~~M~~	~~68~~	~~70~~	~~159~~	~~30~~	~~Choledocotomy~~

TABLE 2
Composition of Lecithin Granules[a]

Phospholipid species (%)	
Phosphatidylethanolamine	31.8
Phosphatidylcholine	24.8
Lysophosphatidylethanolamine	22.1
Lysophosphatidylcholine	8.0
Others	13.3
Fatty acyl chains (%)	
Palmitic acid (16:0)	19.2
Stearic acid (18:0)	4.0
Oleic acid (18:1)	7.9
Linoleic acid (18:2)	53.9
Linolenic acid (18:3)	6.2
Others	8.8

[a]From Lucas Meyer (Hamburg, Germany). Lipids were extracted with chloroform/methanol (23). Phospholipids were separated by thin-layer chromatography as described in the Materials and Methods section. The phospholipids spots were collected, and the phosphorus content was determined (26). Fatty acids composition was determined as described in the Materials and Methods Section.

packed erythrocytes were kept up to four weeks at –20°C for lipid analysis. RBC concentration was determined by a coulter counter (H*1 Analytical console assay; Technicon Instrument Corporation, Tarrytown, NY). Two 0.5 mL aliquots of the packed RBC were taken for lipid extraction. The RBC membrane lipids were extracted according to the method described by Rose and Oklander (30), which demonstrated complete recovery of both FC and phospholipids. FC in the lipid extracts was analyzed enzymatically (Boehringer Mannheim GmbH). Inorganic phosphate in the lipid extracts was eliminated by washing with 0.2 vol of 0.05 M KCl. The washed lipid extracts were then analyzed for phospholipid phosphorus by the method of Bartlett (26).

Analysis of bile and RBC phospholipid fatty acid composition. Aliquots of bile and RBC membrane lipid extracts were saponified with 0.5 M methanolic–KOH at 50°C, and the fatty acids were recovered after acidification by hexane extraction. The fatty acids were methylated with diazomethane and separated by gas–liquid chromatography on a 25 M fused silica column cross-linked with methylsilicone (0.52 mm film thickness) at a temperature range of 180–240°C, employing a Hewlett-Packard (Avondale, PA) 5790A gas-chromatograph equipped with a flame-ionization detector (31). The relative composition of a fatty acid mixture was calculated by a Hewlett-Packard 3390A integrator. For quantitative analysis, heptadecanoic acid was added as internal standard, assuming that the response of the detector for all methyl-fatty acids was identical.

Statistical analysis. In order to compare mean values of bile and plasma lipid concentrations, as well as fatty acid compositions at the various time points, analysis of variance was performed using the multiple comparisons option of Duncan. Because of sample size limitations, a nonparametric test of Kruskal-Wallis was also done. P values equal or less than 0.05 were considered significant.

RESULTS

The biliary lipid composition of the patients throughout the study period is presented in Table 3. Phospholipid supplementation did not cause significant changes in the concentration of the biliary lipid constituents, nor in the distribution of cholesterol between micelles and vesicles. The nucleation time of all samples was long (>10 d) and remained so, irrespective of the diet. Liver function tests of the patients remained within normal limits throughout the study and the follow-up.

The mean plasma phospholipid, cholesterol (free and esterified), and triglyceride levels were 237 ± 40, 50 ± 5, 225 ± 35, and 168 ± 68 mg/dl, respectively, at baseline. These values remained unaffected by phospholipid supplementation (244 ± 44, 50 ± 7, 213 ± 27, and 176 ± 58 mg/dl, respectively).

TABLE 3
Bile Composition[a]

Subject	Sample[a,b]	Ch (mM)	PL (mM)	BS (mM)	CSI	NT(d)	Ch-Ves (%)	Ch-Mic (%)
LM	A	1.84 ± 0.05	8.47 ± 0.45	45.80 ± 12.0	0.77 ± 0.10	>30	3.7 ± 1.6	96.3 ± 1.6
	B	2.26 ± 0.29	8.99 ± 0.27	35.99 ± 3.19	0.97 ± 0.17	28	6.1 ± 0.1	93.9 ± 0.1
	C	2.79 ± 0.22	7.18 ± 0.13	33.78 ± 3.42	1.46 ± 0.10	21	5.0 ± 0.8	95.0 ± 0.8
TN	A	3.27 ± 0.08	10.73 ± 0.76	28.85 ± 1.75	1.29 ± 0.18	18	6.7 ± 3.8	93.3 ± 3.2
	B	3.62 ± 0.52	7.49 ± 0.72	27.05 ± 0.95	1.92 ± 0.14	10	3.5 ± 1.5	96.5 ± 1.5
	C	4.37 ± 0.29	9.35 ± 1.25	28.15 ± 1.35	1.88 ± 0.18	>30	15.2 ± 6.5	84.7 ± 6.5
WE	A	2.91 ± 0.01	8.89 ± 0.39	29.60 ± 3.35	1.33 ± 0.13	>30	1.2 ± 0.2	98.8 ± 0.2
	B	2.66 ± 0.04	8.77 ± 0.98	29.79 ± 6.81	1.26 ± 0.32	>30	1.4 ± 0.5	94.1 ± 4.0
	C	2.71 ± 0.08	8.08 ± 0.88	22.50 ± 0.70	1.53 ± 0.32	>30	7.0 ± 2.2	93.0 ± 2.2
XJ	A	4.55 ± 0.04	14.43 ± 1.43	28.35 ± 4.95	1.50 ± 0.35	>30	15.6 ± 4.2	84.5 ± 4.4
	B	3.97 ± 0.07	16.07 ± 1.03	29.75 ± 0.35	1.20 ± 0.01	>30	26.0 ± 7.5	74.0 ± 7.5
	C	4.65 ± 0.80	13.85 ± 1.14	26.85 ± 0.25	1.57 ± 0.27	>30	21.3 ± 5.2	78.8 ± 5.2
	B*	4.76 ± 0.24	16.34 ± 0.64	37.70 ± 4.70	1.22 ± 0.23	>30	13.5 ± 9.9	86.5 ± 9.9
OA	A	2.19 ± 0.85	5.74 ± 2.48	35.40 ± 2.10	1.40 ± 0.14	>30	10.6 ± 1.1	89.4 ± 1.1
	B	4.52 ± 0.68	15.36 ± 3.00	43.21 ± 0.68	1.15 ± 0.00	>30	6.2 ± 0.4	63.8 ± 3.0

[a]Ch, cholesterol; PL, phospholipids; BS, bile salts; CSI, cholesterol saturation index; NT, nucleation time; Ch-Vis, cholesterol in vesicles; Ch-Mic, cholesterol in micelles.

[b]A, On basal diet; B, after 14 d of phospholipid supplementation; C, 14 d after cessation of phospholipid supplementation; B*, after repeat 14 d of phospholipid supplementation.

TABLE 4
The Relative Fatty Acid Composition (wt% total fatty acids) of Biliary Phospholipids

Subject	Sample[a]	16:0	18:0	18:1	18:2	18:3	20:4	22:6
LM	A	34.0	5.5	10.9	30.9	0.4	6.4	4.1
	B	31.9	5.8	10.3	38.3	0.4	5.7	3.3
	C	35.5	6.0	11.1	31.9	0.5	5.4	4.3
TN	A	34.1	5.3	10.1	29.3	0.6	10.7	1.8
	B	35.3	5.8	11.0	32.8	1.0	9.3	2.1
	C	36.0	5.3	10.9	31.3	0.6	8.6	2.0
WE	A	35.0	5.7	12.0	31.5	0.9	7.8	4.0
	B	33.1	5.3	9.6	39.0	0.5	6.9	3.1
	C	35.6	6.4	10.8	34.4	0.7	6.6	3.0
XJ	A	36.0	4.9	10.0	32.6	0.8	5.8	3.4
	B	33.6	4.7	7.2	37.8	0.6	5.8	2.7
	C	35.4	4.3	7.5	38.8	0.2	5.2	2.9
	B*	33.6	5.0	7.0	41.5	0.3	5.3	3.2
OA	A	40.5	4.2	14.0	31.0	1.0	5.3	1.6
	B	40.7	3.9	8.5	32.8	0.8	4.0	0.9

[a]Sample definitions as in Table 3.

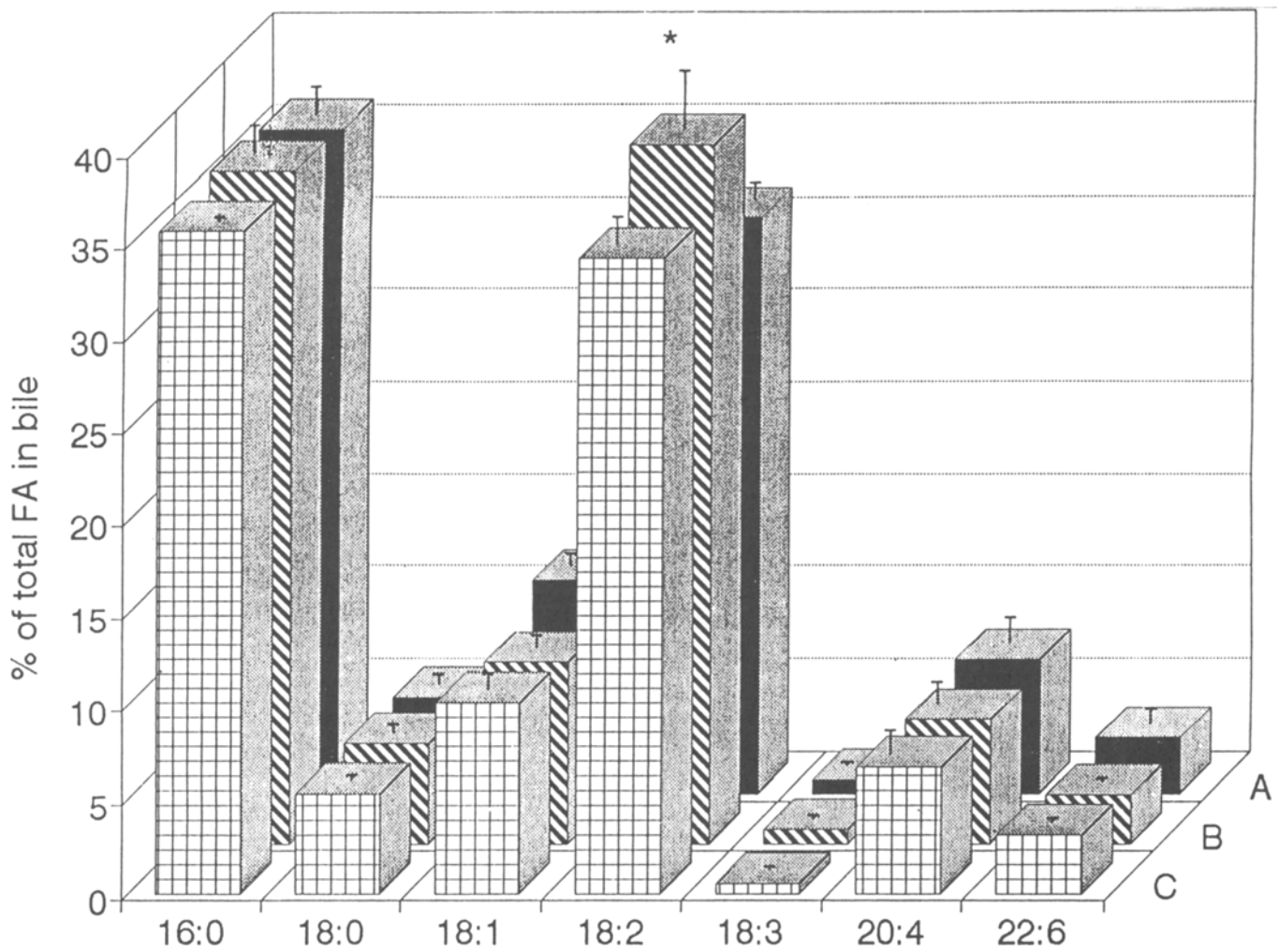


FIG. 1. The fatty acyl profile of biliary phospholipids. The relative percentage (mean ± SD of all five patients) of the main seven biliary fatty acyl species are depicted during the basal diet (A), after 14 d of phospholipid supplementation (B), and 14 d after cessation of phospholipid supplementation (C). *$P < 0.05$ (A and C vs. B).

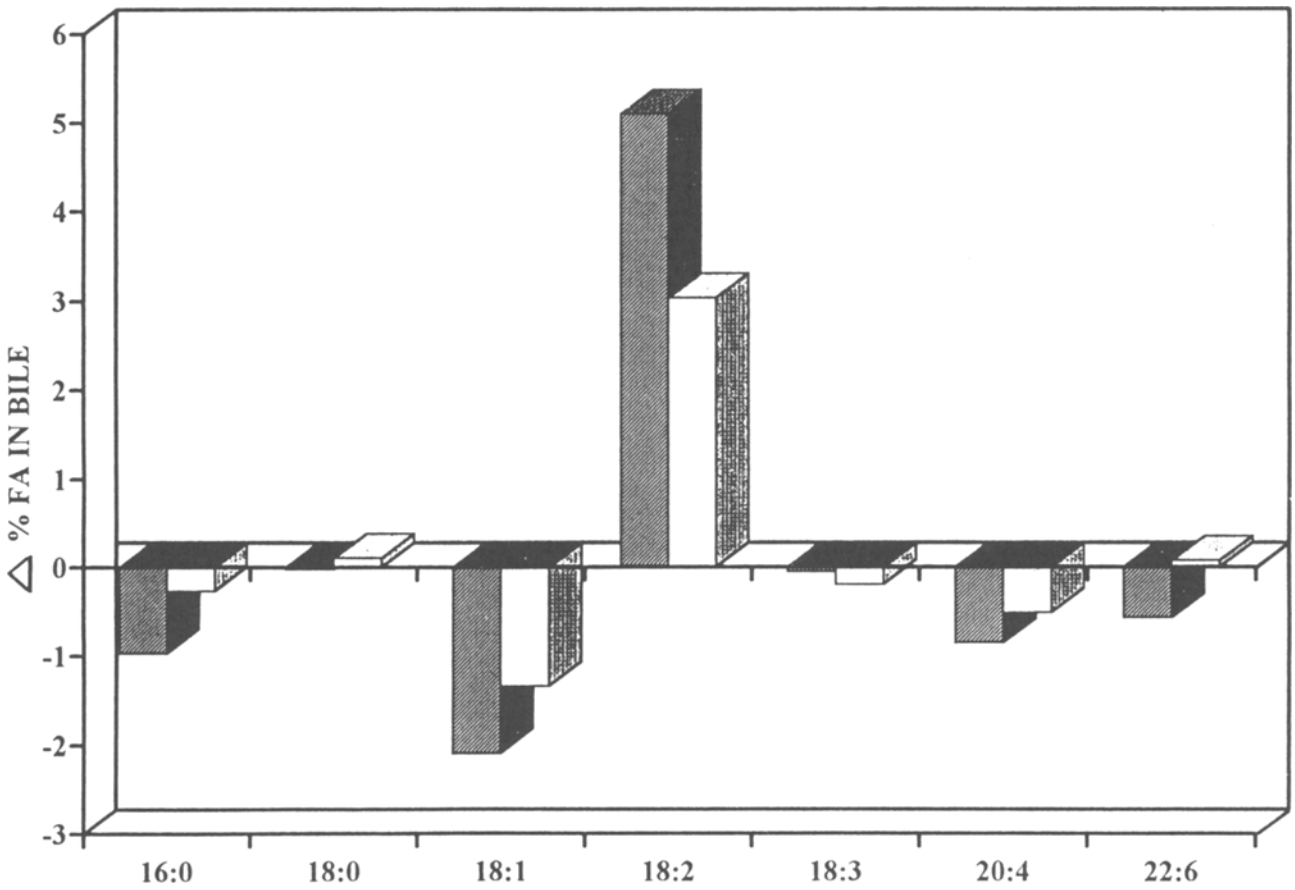


FIG. 2. Relative changes (Δ) in fatty acyl composition of biliary phospholipids. The hatched bars represent changes between the basal diet and phospholipid supplementation. The open bars represent changes between basal diets at the beginning and at the end of the follow-up, i.e., two weeks after phospholipid supplementation. FA, fatty acids.

TABLE 5
Fatty Acid (FA) Composition of Red Blood Cell Membranes (mg/10^{12})

Subject	Sample[a]	Total fatty acids	16:0	18:0	18:1	18:2	20:4	20:0	20:1	22:0	22:1	22:6
XJ	A	85.67	15.74	9.47	8.35	3.47	1.83	7.30	4.80	6.12	2.43	5.86
	B	115.32	28.10	15.59	14.63	5.49	3.07	6.09	3.05	7.66	4.25	6.80
	C	224.81	36.04	23.52	16.99	23.44	21.52	5.09	3.32	5.42	5.83	5.80
	B*	167.81	27.77	20.60	14.17	23.20	18.09	4.30	2.25	5.10	2.56	6.02
OA	A	146.19	31.08	19.28	18.73	8.88	6.80	5.66	2.35	6.76	3.64	6.46
	B	209.36	31.21	27.85	30.19	18.51	25.01	5.74	2.74	6.00	4.80	5.71

[a]Sample definitions as in Table 3.

The main seven biliary phospholipid fatty acyl species are detailed in Table 4 and in Figures 1 and 2. In all five patients, phospholipid supplementation resulted in a significant ($P = 0.05$) enrichment of the bile with linoleic acid (18:2). In Figure 2, which depicts the relative increase/decrease of the means, a marked increase in 18:2 (from 31.1 ± 1.2 to 37.7 ± 5.3%), accompanied by a decrease in 18:1 (from 11.4 ± 1.6 to 9.6 ± 1.1%) can be seen. However, the fatty acid composition started to return to baseline levels when feeding was discontinued. None of the other fatty acyl groups were affected by the treatment. As expected, the total fatty acid concentration remained unchanged. In addition, the relative proportion of PC and PE in bile remained unchanged—the PC/PE ratio in bile was 65.8 ± 25.6 on day 0, 60.6 ± 24.9 on day 14, and 93.4 ± 68.2 on day 28 ($P > 0.4$).

The mean RBC membrane FC and phospholipid concentrations (g/10^{12} cells) were 0.11 ± 0.01 and 0.18 ± 0.04 before, and 0.11 ± 0.00 and 0.21 ± 0.05 after phospholipid sup-

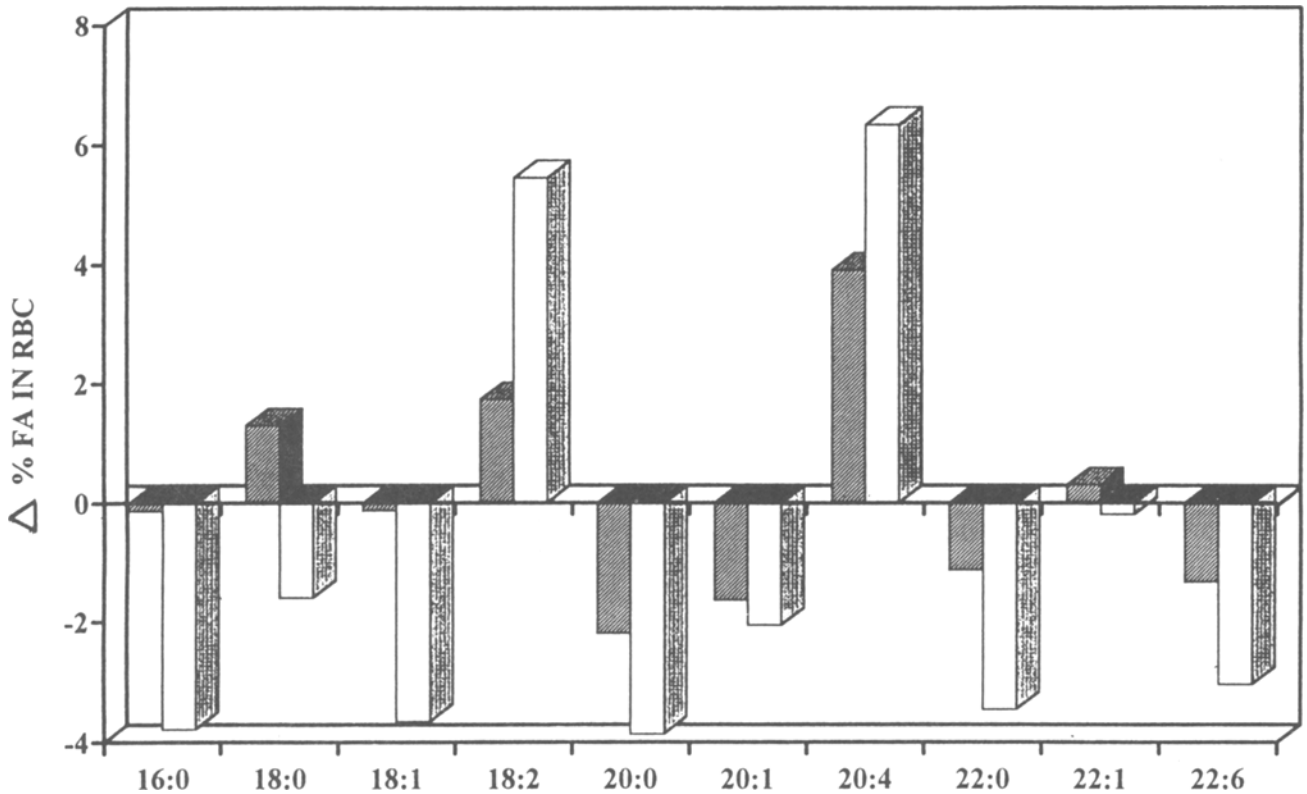


FIG. 3. Relative changes (Δ) in fatty acyl composition of red blood cell membrane phospholipids. The hatched bars represent changes between the basal diet and phospholipid supplementation. The open bars represent changes between basal diets at the beginning and at the end of the follow-up, i.e., two weeks after phospholipid supplementation. FA, fatty acids.

plementation, respectively. Thus, no changes in RBC membrane lipid concentrations or in their relative ratios were detected. The effect of phospholipid supplementation on RBC membrane lipid composition in two patients is detailed in Table 5 and in Figure 3. It can be seen that phospholipid supplementation resulted in marked increases in linoleate (18:2) and arachidonate (20:4) concentrations (from 6.2 to 12.0%, and from 4.3 to 14.0%, respectively). Concomitantly, a slight decrease in the minor fatty acyl species (20:0, 20:1, 22:0, and 22:1) was seen. These effects persisted in patient XJ throughout the prolonged study period, and continued to increase after feeding was discontinued (Fig. 3).

DISCUSSION

Experimental data in humans have revealed differences in biliary lecithin molecular species in gallstone patients as compared to controls. Ahlberg *et al.* (32) found more 16:0–18:1–PC and less 16:0–18:2–PC, whereas others have found increased levels of unsaturated PC acyl chains (especially 20:4) in gallstone patients (33,34). Hay *et al.* (12), on the other hand, failed to find any consistent pattern of lecithin molecular species in cholesterol gallstone patients. Nevertheless, based on experimental evidence, the modulation of phospholipid composition of bile could have very beneficial results. Increasing the relative concentration of phospholipids in human bile retards cholesterol nucleation (7). Changes in phospholipid head groups or in their fatty acyl chains, without any changes in concentration, can produce similar results (8). Unsaturated lecithins accelerate cholesterol crystallization while saturated lecithins retard the process (9,35).

Modulation of biliary phospholipid composition has been accomplished experimentally in laboratory animals (13–16,36), but was insufficiently studied in humans. Feeding a diet rich in legumes reduced the biliary phospholipid concentration in young Chileans (17), whereas dietary fish oils were found to decrease cholesterol saturation in gallstone patients (18). Modulation of biliary phospholipid composition could, in theory, be achieved by changing their hepatic metabolism or their dietary intake. While feeding various phospholipid components to rats and hamsters, it was noted that ethanolamine supplementation resulted in a considerable decrease of cholesterol concentration in bile (19). Bile salts and phospholipids were not significantly affected and the CSI decreased.

About two decades ago, shortly after the introduction of oral bile acid therapy, there were attempts to feed phospholipids to reduce the lithogenicity of bile, but the results were variable and, on the whole, disappointing. Tompkins *et al.* (37) fed 17 patients with 10 g of soybean lecithin for 5–10 d, and collected hepatic bile daily from an open T-tube, begin-

ning on the third post-operative day. They found a significant increase in the phospholipid/cholesterol ratio during phospholipid feeding. However, the relevance of these findings is questionable, because unlike our study, theirs was done with a disrupted enterohepatic circulation. Holan *et al.* (38) fed patients having radiolucent gallstones with 4.5 g of lecithin for three weeks, and examined their duodenal biles at the beginning and the end of the study. They found no significant changes in biliary lipid composition or cholesterol saturation. They did, however, note an increase in 18:2 and, to a lesser extent, in 20:4 fatty acyl chains of phospholipids in their aspirates, suggesting an effect on bile. These early data are consistent with the findings of our study, which examined the effects of phospholipid feeding sequentially in pure bile, under strictly controlled conditions. The efficacy and safety of various phospholipid components in humans were not sufficiently tested. We started our investigation with whole phospholipids, which are normal dietary constituents. The preparation used (Lecithin granules) has been used as a food supplement for many years in several European countries and in Israel. We chose this preparation because of its high PE content (approximately 54%) and record of safety. The amount used, 2 tablespoons three times in day with meals (36 g/d), is considerably larger than that used in previous studies (37,38), and is close to the maximal dose compatible with patient compliance over a period of weeks. All patients had an intact and stable enterohepatic circulation with easy access to repeated bile sampling, due to a chronic indwelling, closed, T-tube. This permitted repeated sampling over a period of several weeks on a fixed diet with and without phospholipid supplementation. In this initial study, we chose to investigate T-tube rather than gallbladder bile, because the former provides a more controlled system. Using T-tube bile, the subjects can serve as their own controls, and the effects of gallbladder function, as well as variations in bile sampling, are avoided (39,40). Moreover, it has recently been shown that metastability and lithogenicity can also be measured in hepatic bile (41).

In our study, the nucleation time, as well as the proportion of cholesterol in phospholipid vesicles, were not altered by the diet. Caution should, however, be exercised in interpreting the results of cholesterol carriers, the quantitation of which is prone to artifacts (42). To overcome some of the possible artifacts, Donovan *et al.* (43) have recently suggested the use of an elution buffer containing the intermicellar concentration of bile salts. However, despite the theoretical justification of this concept, its superiority over the widely used constant bile salt concentration has not been proven when studying human bile. We therefore used the commonly applied and repeatedly tested method of a constant (10 mM NaTC) buffer. This approach was further supported by the fact that the biliary lipid concentrations remained unchanged in each individual patient (who served as their own control) during the study period. Together with the unaffected CSI, our findings of unaltered cholesterol carriers suggest that the diet had no effect on lithogenicity of the bile. One should yet remember that the nucleation time of hepatic bile (as opposed to that of gallbladder bile) may not be a sensitive measure of lithogenicity (41), whereas the CSI certainly is not (44). Phospholipid supplementation did, however, cause a relative enrichment of linoleic acid within biliary phospholipids. This can be explained by the fact that linoleic acid was the main acyl chain (54%) present in the supplementary diet. Whether this enrichment reflects enrichment of the overall phospholipid body pool or maybe more specifically of liver cell membranes is difficult to assess.

The proportion of the essential fatty acid linoleic acid (18:2) increased in bile as well as in RBC. As this fatty acid is not synthesized in the body and its proportion in the ingested phospholipids was high, this result may be expected. Moreover, because 18:2 is rapidly converted to arachidonic acid (20:4), the associated increase in 20:4 can be readily explained. The life span ($T_{1/2}$) of RBC is long (*ca.* 120 d) and they have a limited synthetic capacity after release into the circulation. Because new RBC replace old cells, it is quite logical that the proportion of linoleic acid in RBC increased during dietary supplementation. Furthermore, exchange of membrane phospholipid fatty acids by hydrolysis and reacylation is expected. These increased levels of fatty acids persisting after supplementation were stopped and rose further during a second period of supplementation. In contrast, in the highly labile bile which is secreted *de novo,* the proportion of linoleic acid increased during dietary supplementation and fell rapidly thereafter.

Thus, in the present study, phospholipid, and in particular PE dietary supplementation, failed to change lipid concentrations or lithogenicity of bile. Nevertheless, phospholipid feeding was found to be effective as a vehicle for fatty acyl chain modulation of bile as well as RBC membranes. The pathophysiological relevance of these effects is presently unclear. However, combining the experimental data on the effects of phospholipid species on cholesterol crystallization with the fact that phospholipid species can be enriched in bile prompt further study in this direction. Additional attempts to regulate biliary phospholipids should probably also be directed toward the feeding of phospholipid components or influencing their metabolism in the liver.

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Long-Chain Polyunsaturated Fatty Acids in Plasma Lipids of Obese Children

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ABSTRACT: Fatty acid composition of plasma phospholipids (PL), triglycerides (TG), and sterol esters (STE) was determined by high-resolution capillary gas–liquid chromatography in 22 obese children (age: 13.7 ± 1.4 y, body weight relative to normal weight for height: 170 ± 24%, mean ± SD) and compared with data obtained in 25 age-matched healthy controls. There were no differences in the levels of linoleic acid (LA, $C_{18:2n-6}$) in any of the plasma fractions from the obese children and the controls. Obese children exhibited significantly higher values of arachidonic acid (AA, $C_{20:4n-6}$) than controls both in PL (12.6 [2.4] vs. 8.3 [1.4], % wt/wt, [median (interquartile range)], $P < 0.001$) and STE (7.3 [1.8] vs. 6.0 [1.1], $P < 0.05$). Similarly, obese children showed higher values than controls for dihomo-γ-linolenic acid (DHGLA, $C_{20:3n-6}$) in PL (4.0 [0.5] vs. 3.0 [0.6], $P < 0.001$), TG (0.4 [0.1] vs. 0.2 [0.1], $P < 0.001$), and STE (0.9 [0.1] vs. 0.7 [0.1], $P < 0.01$), and for γ-linolenic acid ($C_{18:3n-6}$) in STE (1.1 [0.2] vs. 0.8 [0.2], $P < 0.001$). The AA/LA ratios were higher in obese children than in controls in PL (0.68 [0.16] vs. 0.42 [0.09], $P < 0.0005$) and STE (0.16 [0.04] vs. 0.12 [0.02], $P < 0.05$), whereas the AA/DHGLA ratios were lower in TG of obese children than in controls (3.40 [0.64] vs. 5.10 [1.75], $P < 0.005$). Plasma glucose concentrations were inversely related to AA in TG ($r = 0.53$, $P < 0.05$), and plasma TG concentrations were inversely related to AA in PL and STE ($r = -0.49$, $P < 0.05$ and $r = -0.48$, $P < 0.05$) and to the AA/DHGLA ratios in PL ($r = -0.57$, $P < 0.01$), TG ($r = -0.56$, $P < 0.01$), and STE ($r = -0.56$, $P < 0.01$). We conclude that the significantly higher values of n-6 long-chain polyunsaturated fatty acids (LCP) in plasma lipids of obese children than in age-matched controls may be caused by an enhanced activity of Δ6-desaturation, and we speculate that elevated fasting immunoreactive insulin seen in obese children (19.4 ± 8.0 μU/mL) may stimulate synthesis of n-6 LCP fatty acids.

Lipids 31, 305–311 (1996).

Obesity is characterized by excessive expansion of adipose tissue mass and multiple derangements of carbohydrate and lipid metabolism. Because disturbances of lipid metabolism are associated with increased risks of morbidity and mortality in obesity, lipid metabolism has been studied extensively in obese subjects. Awareness of the importance of long-chain polyunsaturated fatty acid (LCP) metabolism in obesity has increased considerably in recent years, and fatty acid composition of plasma lipid classes has been investigated in obese adults (1–4). The primary aim of these studies was to examine the effect of weight reduction on fatty acid composition of plasma lipids; however, the most striking finding was a low percentage contribution of the principal n-6 LCP fatty acid, arachidonic acid ($C_{20:4n-6}$, AA), to plasma phospholipids (PL) in untreated obese adults on omnivorous diets (3). Because restriction of dietary fat intake is a cornerstone in the treatment of obesity, alterations of essential fatty acid (EFA) metabolism in obese subjects prior to any dietary intervention raised important questions about the adequacy of weight reduction diets in obesity.

Childhood obesity carries not only an increased risk of lasting morbidity, but also physical and psychosocial burdens, particularly around puberty when many obese children embark on dietary treatment in parallel with the peripubertal growth spurt. If obese children have reduced long-chain polyunsaturated fatty acids (PUFA) prior to dietary treatment, as reported for obese adults (3), dietary reduction of fat intake might worsen EFA status during a time of increased need for EFA for tissue storage and growth. In preterm infants, low n-6 LCP fatty acid values have been associated with reduced growth (5–7).

Controversial results have been reported on the efficacy of n-6 LCP fatty acid synthesis in obese animals. In genetically obese Zucker rats (*fa/fa*), contributions of arachidonic acid to PL in serum, liver, kidney, and heart were lower than in lean (*Fa/-*) animals (8). In contrast, an increase in conversion of linoleic acid (LA, $C_{18:2n-6}$) to AA has been reported in genetically obese mice (9). It is not known whether these animal models represent the pathophysiology of childhood obesity, and whether data obtained in obese adults reflect fatty acid metabolism in childhood obesity. Because we were unable to find data on PUFA in obese children in the published literature, we investigated fatty acid composition of plasma lipid classes in children with untreated obesity.

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Abbreviations: AA, arachidonic acid; DHGLA, dihomo-γ-linolenic acid; EFA, essential fatty acid; GLA, γ-linolenic acid; LA, linoleic acid; IRI, immunoreactive insulin; LCP, long-chain polyunsaturated fatty acid; PL, phospholipid; PUFA, polyunsaturated fatty acids; STE, sterol ester; TG, triglyceride.

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MATERIALS AND METHODS

The investigations were carried out in 22 obese children (11 girls and 11 boys) referred to a consultant pediatrician (D.M.) at the Department of Pediatrics, University Medical School of Pécs (Pécs, Hungary), because of their weight. The children were free of other known illnesses, were not dieting, and were not participating in physical training programs. The study was carried out in accordance with the Declaration of Helsinki II and with approval of the ethics committee of the University Medical School of Pécs. Informed parental consent was obtained for each child before enrollment in the study.

Anthropometric measurements were carried out by the same investigator. Body weight was determined to the nearest 0.5 kg with the child dressed only in underwear and wearing no shoes. Height was measured to the nearest 0.1 cm by a Holtain stadiometer (Holtain Limited, Crymich, Dyfed, Britain). Body fat was estimated according to Parizková and Roth (10) from five skinfold thicknesses measured on the left side of the body three consecutive times with the help of a Holtain caliper. Lean body mass was calculated by subtracting body fat from actual body weight. Relative body weight was calculated as the ratio between actual body weight and the ideal body weight for age, gender, and height (11). The waist and hip circumferences were measured with the subject in the supine position. The pubertal stage of the children was determined according to Tanner (12).

Blood samples were taken from the antecubital vein in the fasting state early in the morning. Blood was collected into tubes containing 2 mg/mL EDTA for triglyceride, cholesterol, plasma glucose, and insulin determinations, and into another tube for fatty acid analysis. The samples were centrifuged immediately at 4°C and plasma was stored at –20°C until analysis. Plasma samples were thawed only once.

Laboratory analyses, except for fatty acid determinations, were carried out at the Department of Pediatrics, Pécs. Plasma glucose concentrations were determined by the glucose oxidase method (13). Plasma insulin concentrations were measured with commercially available radioimmunoassay kits (Isotope Institute of the Hungarian Academy of Sciences, Budapest, Hungary). With this method, the upper limit of the normal range (mean + 2SD) for immunoreactive insulin levels in 100 healthy control children was found to be 20 μU/mL. Triglyceride and cholesterol were determined with an enzymatic kit (Boehringer, Mannheim, Germany).

Fatty acid analysis was carried out at the Kinderpoliklinik of the University of Munich (Munich, Germany). Plasma lipids were extracted from 0.25 mL plasma with chloroform/methanol (14) after addition of internal standards. Lipid classes were separated on silica gel plates (Nr. 5721; E. Merck, Darmstadt, Germany) with one 18-cm run of petroleum ether/ether/acetic acid (75:25:1, by vol), then 5-cm run of chloroform/methanol/water (65:25:4, by vol) (15). Fatty acids in plasma PL, triglycerides (TG), and sterol esters (STE) were transesterified with methanol and hydrochloric acid (16) and analyzed by high-resolution capillary gas–liquid chromatography.

A Hewlett-Packard 5890 (series II) gas chromatograph, equipped with a Sil 88 cyanopropil column of 50-m length (Chrompack, Middelburg, The Netherlands), was used to identify the fatty acid methyl esters. The running conditions for these determinations were: initial temperature, 60°C, followed by an increase (20°C/min) to 190°C for 1 min, followed by another increase (25°C/min) to 230°C for 31 min. The column head pressure was 1.5 bar. Identification of fatty acids was verified by comparison with authentic standards (Nu-Chek-Prep, Elysian, MN) and mass spectrometry (Hewlett-Packard Series 5971 MSD). Fatty acid results were expressed as percentage (w/w) of all fatty acids detected with a chain length between 12 and 22 carbon atoms.

The normal plasma fatty acid composition values used for comparison in this study were taken from a previously published study involving a sizable cohort (n = 115) of healthy children (17). From this data pool, data of the subgroup of children aged 10 to <15 years (n = 25, 13 males and 12 females, mean age: 12.9 ± 1.6 yr, mean ± SD) were used for comparison. These children were investigated either because of symptoms presumably not related to fatty acid metabolism, or because of planned elective surgery calling for general anesthesia. One of the inclusion criteria in the study was body weight between the 3rd and 97th centiles. Fasting plasma was obtained from these subjects and analyzed for fatty acid composition with procedures identical to those described above.

Results were evaluated with Minitab for Windows, Release 9 (18). All data except fatty acids were compared by Student's unpaired *t*-test. The fatty acid data are presented as median and interquartile range values; because skewed distributions were found, especially present at low concentrations, Mann-Whitney's two-sided rank test was used for intergroup comparison of fatty acid profiles. Differences were regarded as statistically significant at $P < 0.05$.

RESULTS

The subjects' characteristics are shown in Table 1. All subjects were markedly obese; the extent of overweight expressed as a percentage of ideal body weight ranged from 31 to 131%. Age, body weight, relative body weight, height, and body mass index did not differ significantly between boys and girls, though girls tended to be slightly heavier and somewhat shorter than boys (Table 1). Body fat content was significantly higher in girls than in boys. Consequently, lean body masses tended to be higher in boys than in girls; however, the differences did not reach statistical significance. The waist to hip ratio of males was significantly higher than that of females, a finding indicative of a tendency to abdominal adiposity in males. Girls were sexually more mature than boys; median Tanner's stages of public hair/breast development in girls were 5:5, whereas median stages of public hair/genital development in boys were 2:3.

Plasma TG, cholesterol, glucose, and immunoreactive insulin (IRI) concentrations are shown in Table 1. Average TG concentrations were only slightly elevated; when considered individually, seven subjects (4 girls and 3 boys) exhibited hy-

TABLE 1
Characteristics of the Subjects[a]

	Girls (n = 11)	Boys (n = 11)	All (n = 22)
Age (y)	13.9 ± 1.4	13.6 ± 1.5	13.7 ± 1.4
Weight (kg)	89.7 ± 20.3	88.3 ± 22.2	89.0 ± 20.8
RBW (%)	177 ± 25	166 ± 11	170 ± 24
Height (cm)	161 ± 8	166 ± 11	164 ± 10
BMI (kg/m^2)	34.2 ± 5.2	31.7 ± 5.2	32.9 ± 52
BF (%)	43.1 ± 3.0[c]	35.0 ± 4.0[c]	39.1 ± 5.4
LBM (kg)	50.9 ± 11.4	57.0 ± 13.9	54.0 ± 12.8
Waist/hip ratio	0.80 ± 0.08[b]	0.90 ± 0.06[b]	0.85 ± 0.08
Triglyceride[b] (mmol/L)	1.59 ± 0.55	1.69 ± 0.84	1.64 ± 0.70
Cholesterol (mmol/L)	4.11 ± 0.61	4.35 ± 0.60	4.24 ± 0.60
Glucose (mmol/L)	4.11 ± 0.58	4.29 ± 0.76	4.20 ± 0.67
IRI (μU/mL)	21.0 ± 7.8	17.8 ± 8.2	19.4 ± 8.0

[a]Values are mean ± SD. Means in a row sharing a common superscript (b,c) are significant different ([b]$P < 0.005$, [c]$P < 0.0001$) using Student's unpaired *t*-test; RBW, relative body weight; BMI, body mass index; BF, body fat content; LBM, lean body mass; IRI, fasting immunoreactive insulin.
[b]Cutoff points of hypertrigliceridemia and hypercholesterolemia in children were set by the Hungariank Lipid-Consensus Conference as 1.6 and 5.2 mmol/L, respectively.,

pertriglyceridemia with serum TG concentrations > 1.6 mmol/L (range: 1.8–3.9 mmol/L). Plasma cholesterol concentrations were not markedly elevated; the maximal value was 5.3 mmol/L. In spite of normoglycemia (range of plasma glucose concentrations: 2.3–5.0 mmol/L), basal plasma IRI concentrations exceeded the value for normal children in nine subjects (5 girls and 4 boys; range: 21–39 μU/mL). Basal plasma immunoreactive insulin concentrations tended to be higher in girls than in boys; the mean value for the IRI levels in the obese girls was greater (by 1 μU/mL) than the upper limit of normality (mean + 2 SD) reported for healthy females.

The fatty acid composition of plasma lipid classes in the obese children is compared with values of age-matched controls in Table 2. There were no significant differences between boys and girls in percentage contributions of fatty acids to plasma PL, TG, and STE either in the present investigation, or in a previous study on healthy children (18); therefore, pooled values for both sexes are shown in Table 2. Percentage contributions of palmitic acid ($C_{16:0}$) in the TG and STE fractions and stearic acid ($C_{18:0}$) in the STE fraction were significantly higher in the obese children compared with the controls. Total saturated fatty acids were significantly higher in TG and STE of obese children than in controls. In plasma PL, however, only stearic acid ($C_{18:0}$) values were elevated in the obese subjects, whereas levels of myristic ($C_{14:0}$) and palmitic ($C_{16:0}$) acids were highest in the control group. Total saturated fatty acids in PL did not differ between the two groups. Octadecenoic acids, that is, oleic ($C_{18:1n-9}$) plus vaccenic ($C_{18:1n-7}$) acid, in the PL fraction and total monounsaturated fatty acids in the PL and TG fractions were significantly lower in obese than in the control children.

Percentage contributions of the n-6 precursor essential fatty acid LA, as well as percentage contributions of eicosadienoic acid ($C_{20:2n-6}$), did not differ between the two groups. In contrast, obese children had significantly higher levels of

TABLE 2
Major Fatty Acids of Plasma Lipid Classes in Obese Children and in Age-Matched Controls[a]

Fatty acid	Phospholipids		Triglycerides		Sterol esters	
	Obese	Control	Obese	Control	Obese	Control
Saturated fatty acids						
$C_{14:0}$	0.28(0.07)[d]	0.52(0.12)[d]	1.40 (0.37)	1.64 (0.43)	0.69 (0.10)	0.82 (0.59)
$C_{16:0}$	27.24 (1.74)[b]	28.71 (2.00)[b]	28.14 (1.44)[c]	25.80 (1.84)[c]	13.24 (0.63)[c]	11.71 (0.87)[c]
$C_{18:0}$	17.41 (1.27)[b]	16.34 (1.44)[b]	4.24 (0.60)	4.23 (0.22)	1.83 (0.25)[c]	1.39 (0.27)[c]
Total	46.04 (0.95)	45.71 (1.98)	34.09 (1.95)[b]	31.45 (3.26)[b]	15.94 (0.91)[c]	14.54 (1.56)[c]
Monounsaturated fatty acids						
$C_{16:1n-7}$	0.48 (0.09)	0.55 (0.12)	3.55 (0.59)	3.59 (1.09)	3.18 (0.51)	3.38 (0.67)
$C_{18:1n-9/7}$	11.08 (0.89)[d]	13.72 (1.26)[d]	39.92 (2.33)	42.93 (2.34)	21.93 (2.09)	20.38 (1.73)
Total	12.38 (1.22)[d]	15.19 (1.35)[d]	44.55 (1.95[b]	48.00 (2.48)[b]	25.67 (2.51)	24.26 (1.71)
n-6 Polyunsaturated fatty acids						
$C_{18:2n-6}$	19.79 (2.33)	20.24 (2.32)	16.29 (2.39)	14.14 (1.97)	47.66 (3.56)	49.06 (3.43)
$C_{18:3n-6}$	0.07 (0.04)	0.07 (0.04)	0.25 (0.07)	0.26 (0.10)	1.07 (0.22)[d]	0.77 (0.21)[d]
$C_{20:2n-6}$	0.39 (0.06)	0.37 (0.04)	0.22 (0.04)	0.23(0.06)	ND	0.06 (0.02)
$C_{20:3n-6}$	3.97 (0.51)[d]	2.96 (0.62)[d]	0.41 (0.08)[d]	0.20 (0.08)[d]	0.92 (0.14)[c]	0.68 (0.12)[c]
$C_{20:4n-6}$	12.63 (2.41)[d]	8.34 (1.44)[d]	1.29 (0.31)	1.05 (0.07)	7.27 (1.85)[b]	6.01 (1.08)[b]
$C_{22:4n-6}$	0.57 (0.08)[d]	0.39 (0.10)[d]	0.19 (0.04)[c]	0.11 (0.07)[c]	ND	ND
Σ n-6 LCP[b]	18.15 (1.84[d]	12.34 (1.78)[d]	2.32 (0.40)[c]	1.62 (0.38)[c]	8.24 (1.77)[b]	6.72 (1.02)[b]
n-3 Polyunsaturated fatty acids						
$C_{18:3n-3}$	ND	0.15 (0.04)	0.26 (0.06)[d]	0.48 (0.09)[d]	0.16 (0.06)[d]	0.51 (0.05)[d]
$C_{22:5n-3}$	0.57 (0.08)	0.79)0.44)	0.16 (0.04)	0.35 (0.21)	ND	ND
$C_{22:6n-3}$	2.65 (0.67)	2.46 (0.46)	0.31 (09.07)	0.36 (0.17)	0.50 (0.30)	0.61 (0.15)
Σ n-3 LCP[c]	3.40 (0.60)	3.68 (1.13)	0.47 (0.07)[b]	0.72 (0.38)[b]	0.55 (0.30)	0.66 (0.13)

[a]Values are % w/w [median (interquartile range)], obese: n = 22, control: n = 25, ND denotes not detectable. Medians in a row sharing a common superscript (b,c,d) are significantly different ([b]$P < 0.05$, [c]$P < 0.01$, [d]$P < 0.001$) using Mann-Whitney's two-sided rank test.
[b]Total n-6 long-chain polyunsaturated fatty acid (LCP) denotes ($C_{20:2n-6} + C_{20:3n-6} + C_{20:4n-6} + C_{22:4n-6}$).
[c]Total n-3 LCP denotes ($C_{20:5n-3} + C_{22:5n-3} + C_{22:6n-3}$).

TABLE 3
Product/Substrate Ratios of n-6 Long-Chain Polyunsaturated Fatty Acid Biosynthesis in Plasma Lipid Classes of Obese Children and Age-Matched Controls[a]

Ratio	Phospholipids		Triglycerides		Sterol esters	
	Obese	Control	Obese	Control	Obese	Control
$(C_{18:3n-6} + C_{20:3n-6})/C_{18:2n-6}$	0.21 (0.06)[d]	0.15 (0.04)[d]	0.04 (0.01)	0.03(0.01)	0.04 (0.01)[a]	0.03 (0.01)[d]
$C_{20:4n-6}/C_{20:3n-6}$	3.08 (0.56)	2.85 (0.71)	3.40 (0.64)[c]	5.10 (1.75)[c]	8.41 (1.70)	9.05 (1.81)
$C_{20:4n-6}/C_{18:2n-6}$	0.68 (0.16)[d]	0.42 (0.09)[d]	0.08 (0.02)	0.07 (0.01)	0.16 (0.04)[b]	0.12 (0.02)[b]

[a]Values are [median (interquartile range)], obese: n = 22, control: n = 25. Medians in a row sharing a common superscript (a,b,c,d) are significantly different ([b]$P < 0.05$, [c]$P < 0.005$, [d]$P < 0.0005$) using Mann-Whitney's two-sided rank test.

the metabolites of Δ6-desaturase, γ-linolenic acid (GLA, $C_{18:3n-6}$) in the STE fraction, dihomo-γ-linolenic acid (DHGLA, C20:3n-6) in all of the plasma fractions, AA in the PL and STE fractions and adrenic acid ($C_{22:4n-6}$) in the PL and TG fractions than the controls (Table 2).

Percentage contributions of the n-3 precursor essential fatty acid, α-linolenic acid ($C_{18:3n-3}$), were significantly lower in all of the plasma fractions in obese children than in controls. There were no differences between the two group in the percentage contributions of the principal n-3 LCP fatty acid, docosahexaenoic acid ($C_{22:6n-3}$), but total n-3 LCP fatty acids were significantly lower in TG of obese than in controls.

Product/substrate ratios for n-6 LCP fatty acid biosynthesis in obese subjects are compared with data of controls in Table 3. The ratios of GLA plus DHGLA to LA, i.e., the product/substrate ratios reflecting the Δ6-desaturase enzyme activity, were higher in both PL and STE in obese subjects than in controls. In contrast, the ratios of AA to DHGLA ($C_{20:4n-6}/C_{20:2n-6}$) were significantly lower in TG of obese children than in controls, whereas no differences in PL and STE were seen. The ratios of AA to LA ($C_{20:4n-6}/C_{18:2n-6}$) were significantly higher in both PL and STE of obese subjects than in controls. No significant differences were found when the product/substrate ratios calculated for girls were compared with those calculated for boys.

The twelve characteristics of the subjects (age, weight, height, relative body weight, body fat content, lean body mass, body mass index, waist to hip ratio, plasma glucose, IRI, cholesterol, and TG concentrations) were used as predictive variables in multiple linear regression analyses, whereas percentage contributions of AA and the AA/LA and AA/DHGLA ratios in plasma lipid classes were entered as responding variables. Significant inverse correlations were seen between plasma TG concentrations and percentage contributions of AA in plasma PL ($r = -0.45$, $P = 0.04$) and STE ($r = -0.48$, $P = 0.03$). A significant inverse correlation existed between plasma glucose concentrations and the percentage contribution of AA in plasma TG ($r = -0.53$, $P < 0.05$). Similarly, the ratios of AA to LA in TG ($C_{20:4-6}/C_{18:2n-6}$) were inversely correlated with plasma glucose concentrations ($r = -0.61$, $P < 0.005$). In both PL and STE, the AA/LA ratios were inversely related to plasma TG concentrations (r −0.51, $P < 0.05$; $r = -0.48$, $P < 0.05$). Body heights were significantly correlated with the AA/LA ratio in STE ($r = 0.44$, $P < 0.05$). The ratios of AA to DHGLA ($C_{20:4n-6}/C_{20:3n-6}$), i.e., the substrate/product ratios for Δ5-desaturase enzyme were inversely related to the plasma TG concentrations in all three lipid classes investigated (PL: $r = -0.57$, $P < 0.01$; TG: $r = -0.56$, $P < 0.01$; STE: $r = -0.56$, $P < 0.01$).

Amounts of substrate ($C_{20:3n-6}$) and product ($C_{20:4n-6}$) of the Δ5-desaturase and the $C_{20:4n-6}/C_{20:3n-6}$ ratio in obese children with plasma TG concentrations > 1.6 mmol/L) were compared with the values from children with plasma TG concentrations ≤1.6 mmol/L (Table 4). In all lipid classes investigated, percentage contributions of DHGLA ($C_{20:3n-6}$) were similar in the two groups, but children with hypertriglyceridemia tended to have lower AA ($C_{20:4n-6}$) values than children with <1.6 mmol/L plasma TG concentrations. The AA/DHGLA ratios were significantly lower in the children with plasma TG concentrations >1.6 mmol/L than in those with ≤1.6 mmol/L plasma TG concentrations (Table 4).

No significant differences were found in percentage contributions of n-6 LCP fatty acids and their product/substrate ratios in children with and without basal hyperinsulinemia (IRI concentrations > 20 μU/mL) (data not shown).

DISCUSSION

In the present study, we compared the fatty acid composition of plasma lipid classes in obese children with the corresponding values obtained in a sizable cohort of healthy children, and the fatty acid data were analyzed in relation to parameters characterizing the obese subjects. In contrast to previous observations in obese adults compared with nonobese controls (3,19), we found that percentage contributions of n-6 LCP fatty acids to plasma lipid classes were higher in obese children than in age-matched control subjects. This finding raises the question of determining which mechanisms may have contributed to enhanced n-6 LCP fatty acid values in obese children.

High percentage contributions of n-6 LCP fatty acids to plasma lipid classes may arise as the result of at least five different factors: (i) a high dietary intake of preformed n-6 LCP; (ii) a high dietary intake of LA ($C_{18:2n-6}$); (iii) enhanced rate of the synthesis of n-6 LCP fatty acids from LA; (iv) diminished rate of n-6 LCP fatty acid utilization (for incorporation into tissues, eicosanoid synthesis and oxidation); and 5) increased liberation of stored LCP fatty acids from body pools (mainly from adipose tissues).

We have no detailed information on the diet of the children

TABLE 4
Percentage Contributions of n-6 Substrate ($C_{20:3n-6}$) and Product ($C_{20:4n-6}$) of Δ5-Desaturase in Obese Children with and without Plasma Triglyceride Concentrations >1.6 mmol/L[a]

Plasma triglyceride	>1.6 mmo/L	≤1.6 mmoll/L
	(n = 7)	(n = 15)
Plasma triglyceride concentration (mmol/L, mean ± SD)	2.43 + 0.68[a]	1.25 ± 0.20[a]
Fatty acids [(% w/w, median (interquartile range)]		
Plasma phospholipids		
$C_{20:3n-6}$ (% w/w)	3.99 (0.88)	3.81 (0.59)
$C_{20:4n-6}$ (% w/w)	10.78 (1.21)	13.69 (1.72)
$C_{20:4n-6}/C_{20:3n-6}$	2.60 (0.50)[b]	3.47 (0.67)[b]
Plasma triglycerides		
$C_{20:3n-6}$ (% w/w)	0.44 (0.07)	0.39 (0.10)
$C_{20:4n-6}$ (% w/w)	1.02 (0.21)	1.36 (0.25)
$C_{20:4n-6}/C_{20:3n-6}$	2.67 (0.74[b]	3.58 (0.68)[b]
Plasma sterol esters		
$C_{20:3n-6}$	1.00 (0.07)	0.85 (0.17)
$C_{20:4n-6}$	6.12 (1.28)	8.45 (1.53)
$C_{20:4n-6}/C_{20:3n-6}$	6.03 (1.30)[b]	9.07 (1.50)[b]

[a]Means in a row sharing a common superscript are significantly different ([a]$P < 0.005$) using Student's unpaired *t*-test. Medians in a row sharing a common superscript are significantly different ([b]$P < 0.05$) using Mann-Whitney's two-sided rank test.

prior to the study; however, enhanced dietary intake of fatty acids (either LCP or precursor) seems to be an unlikely explanation for the elevated n-6 LCP values observed in the obese children. In a previous study in obese children from this same population, it was found that they did not consume more fat than nonobese controls, either when fat intake is expressed as g/kg body weight or g/kg lean body mass (20). Furthermore, there are few foods in which AA constitutes more than 2–6% of the total fatty acids by weight, and these foods, including such items as egg yolk, beef liver, mackerel, and whale oils (21), are unlikely to contribute substantially to the diet of young teenagers. Consequently, the fact that the obese children originated from a geographically different population than the controls could not have a major influence on dietary intake of preformed n-6 LCP such as AA. A high dietary intake of LA would have been reflected also in high percentage contributions of LA to plasma lipid classes, but LA values in obese children did not differ from those measured in controls (Table 2).

In spite of the similar values for LA ($C_{18:2n-6}$), percentage contributions of GLA ($C_{18:3n-6}$), DHGLA ($C_{20:3n-6}$), and AA ($C_{20:4n-6}$) were significantly higher in the obese children than in the controls. This pattern is consistent with an enhanced conversion of LA to the other n-6 LCP fatty acids. The finding that percentage contributions of eicosadienoic acid ($C_{20:2n-6}$), a direct elongation product of LA, did not differ between the obese and nonobese groups, supports the hypothesis that chain-elongation was unaffected but desaturase activity was enhanced in the obese children. The product/substrate ratios calculated for the enzymes of n-6 LCP fatty acid synthesis support this concept further.

The substrate/product ratios for the Δ6-desaturase ($[C_{18:3n-6} + C_{20:3n-6}]/C_{18:2n-6}$) were significantly higher in both PL and STE of obese children than in controls. In plasma TG, this ratio did not differ significantly between the two groups. The substrate/product ratios for the Δ5-desaturase ($C_{20:4n-6}/C_{20:3n-6}$) did not differ between the two groups in PL and STE; at the same time, obese children exhibited significantly lower values in TG. In conclusion, our data are consistent with the hypothesis that enhanced activity of the Δ6-desaturase contributed to higher AA and n-6 LCP fatty acid values in plasma lipids in obese children. This hypothesis is further supported by the absence of other known pathophysiological mechanisms, such as diminished n-6 LCP utilization or increased liberation of n-6 LCP from body stores in nondieting obese teenagers.

There are several nutritional and hormonal factors that play a role in the regulation of essential fatty acid desaturation (22,23) and which may have figured in the enhanced Δ6-desaturase activity seen in the obese children in this study. In animal experiments Δ6-desaturase is stimulated by protein and energy intakes (24,25), and low Δ6-desaturase activity has been suggested as the mechanism for the low AA and n-6 LCP fatty acid values seen in children with protein-energy malnutrition (26–28).

Insulin has been reported to be a potent activator of Δ6-desaturase (24,29,30,31), and the insulin secreted by obese individuals has full biological activity (32). Even though we found no significant correlation between insulin concentrations and LCP fatty acid values, and fatty acids in children with basal hyperinsulinemia did not differ, we still consider that the action of insulin may explain at least in part the high n-6 LCP fatty acid values observed in the obese children. This hypothesis is further supported by the absence of other known

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Our results in obese children contrast with findings of reduced n-6 LCP in obese adults (3). However, low n-6 LCP fatty acid values were not a unanimous finding in obese adults; Rössner and co-workers (1) reported significantly higher AA values in plasma cholesterol esters in obese adults than in controls. It is possible that differences in the study populations may have contributed to the varying results. Some of the pertinent factors analyzed in the present study (particularly glucose and insulin concentrations) were not reported in investigations on obese adults (1,3). Hyperlipidemia is a common finding in advanced obesity, and significant inverse relations of plasma triglyceride concentrations to product/substrate ratios of enzymes of n-6 LCP synthesis were seen in this study. Cunnane *et al.* (37) reported a similar phenomenon, i.e., an inverse correlation of the percentage contribution of AA in liver triacylglycerols to total triacylglycerol concentrations in rats, hamsters, guinea pigs, and monkeys. Increased AA values are a consistent observation during weight reduction in obese adults (1,3,4) and were also reported in parallel with significant reductions of plasma TG concentrations (1).

In the present study, the product/substrate (AA/DHGLA) ratio was significantly lower in TG of obese children than in controls, which is in agreement with the observation of Guesnet and co-workers (8), who recently reported significantly lower AA/DHGLA ratios of plasma and tissue samples in obese than in lean female Zucker rats.

We hypothesize that the higher n-6 LCP fatty acid values found in obese children are due to an enhancement of the Δ6-desaturase enzyme activity, whereas the Δ5-desaturase activity appeared lower in the obese children than in the controls. Our data provide evidence that the risk of an EFA deficiency is not increased when diets low in the n-6 LCP fatty acids are consumed during the treatment of obesity is adolescents.

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Changes in the Size and Docosahexaenoic Acid Content of Adipocytes During Chick Embryo Development

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ABSTRACT: The development of adipose tissue in the chick embryo was investigated using two groups of fertile eggs which differed by 1.7-fold in their initial yolk lipid levels. The triacylglycerol content of the subcutaneous adipose depot in both groups increased dramatically from day 12 of the 21-day embryonic period, attaining a maximal value just prior to hatching. During this period, the amount of triacylglycerol deposited in the adipose tissue was very highly correlated with the amount of lipid transferred from the yolk. The triacylglycerol content of the depot was also dependent on the initial yolk lipid content. During the hatching period, the amount of adipose triacylglycerol remained approximately constant in the group with the higher initial yolk lipid content but, in the case of the group with the lower initial yolk lipid levels, decreased by approximately 25%. The size distribution of adipocytes isolated from the tissue was determined by computerized image analysis microscopy. The mean adipocyte diameter increased from approximately 6 to 35 μm between days 12 and 19, irrespective of the initial yolk content, although development within the eggs with the lower initial yolk content resulted in a decrease in cell size over the hatching period. Both the triacylglycerol and phospholipid fractions of the isolated adipocytes contained substantial proportions (approximately 6%, w/w) of docosahexaenoic acid (DHA) at days 12 and 14, and lower levels of this fatty acid at the later stages. The amount (mg/depot) of DHA in adipose triacylglycerol decreased dramatically over the hatching period. The amount (mg/brain) of DHA in brain phospholipid increased by more than 5-fold between day 12 of development and hatching. A possible explanation for the data may be that DHA is preferentially mobilized from adipose tissue in order to deliver the fatty acid to the developing neural tissues in a form suitable for uptake. *Lipids 31*, 313–321 (1996).

The final week of the 21-d embryonic period of the chicken is characterized by the rapid transfer of lipid components from the yolk to the embryo (1). Current evidence indicates that yolk lipids are taken up by the yolk sac membrane where they are assembled into lipoprotein particles for release into the embryonic circulation (1,2). The action of lipoprotein lipase, located at various tissue sites, on the plasma lipoproteins results in the uptake and utilization of the constituent fatty acids by the embryonic tissues (3). The resultant lipoprotein remnants are taken up by the embryonic liver, with the consequent accumulation of large amounts of cholesteryl ester in this tissue (4,5).

The intensity of the lipid transfer process, the predominance of lipid metabolism in the embryonic tissues, and the fact that the embryo develops as a self-contained system free from contemporaneous maternal influences (1) are factors which favor the suitability of the chick embryo as a model system for studying various aspects of lipid utilization during development. The present study encompasses the relationship between two such aspects; namely, the development of adipose tissue and the delivery of essential long-chain polyunsaturated fatty acids, in particular docosahexaenoic acid (DHA; 22:6n-3), to the embryonic tissues.

Subcutaneous adipose tissue can be detected in the chick embryo from around day 12 of development, and the tissue then grows rapidly during the final week of embryogenesis (3,6). The fact that rates of lipogenesis from carbohydrate precursors are extremely low in this system (7), coupled with the observation that very high levels of lipoprotein lipase activity are expressed in the embryo's adipose tissue (3), suggests that the growth of the tissue is dependent largely on the uptake of fatty acids hydrolyzed from yolk-derived plasma lipids. The sensitivity of the adipocytes to lipolytic agonists increases toward the end of the embryonic period (8), suggesting that one of the functions of adipose tissue in this system may be to provide a store of triacylglycerol which can be partially mobilized for energy purposes during hatching.

High levels of DHA are incorporated into the phospholipids of the brain and retina of the chick embryo (9,10) and of most other vertebrates (11) during development, and an adequate supply of this fatty acid is deemed to be essential for the optimal functional maturation of these tissues (11). Previous studies from our laboratory have shown that the transport of DHA from the yolk to the embryonic tissues is characterized by a series of unique features, including the preferential uptake of DHA-containing phospholipids from the yolk by the yolk sac membrane (1), the specific transfer of yolk-derived DHA into the triacylglycerol fraction of plasma lipoproteins (3,12), and the incorporation of large proportions of this fatty acid into the triacylglycerols of the liver and adipose tissues (1,3). In the present study, changes in adipocyte size dis-

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tribution and in the DHA content of the adipose tissue lipids were evaluated from the time of the initial formation of the tissue until the end of the embryonic period. Changes in the DHA content of brain phospholipid during the same period are also reported.

MATERIALS AND METHODS

Embryos. Fertile eggs of the Ross 1 broiler-breeder strain were obtained from Ross Poultry Ltd. (Inverurie, United Kingdom) and incubated at 37.8°C and 60% relative humidity in a forced-draught incubator with automatic egg turning. Hatching occurred after 21 d of incubation, and the chicks were maintained in the incubator at 37.8°C and 60% relative humidity for one day after hatching, with free access to drinking water but with no food provision. In order to compare the effects of differences in the lipid provision from the yolk on the development of adipose tissue in the embryo, two experiments were performed using batches of eggs which differed in their initial yolk lipid content. The initial egg weights (g) for the two batches were 48.4 ± 0.7 and 62.2 ± 0.7 (means ± SEM, measured on 20 eggs from each batch), and the initial lipid contents of the yolks (g total lipid/yolk) were 3.4 ± 0.1 and 5.7 ± 0.1 (means ± SEM, measured on 8 eggs from each batch). The respective DHA contents (mg/yolk) of the initial yolk lipid samples were 67.4 ± 2.2 and 94.3 ± 4.1 (means ± SEM measured on 4 eggs from each batch). At the appropriate developmental stages, embryos were excised from the eggs, and the subcutaneous adipose tissue was quantitatively dissected from the upper thigh area and along the sides of the abdomen and thorax. The combined tissue from these sites from both sides of a single embryo is hereafter referred to as a depot. Other, more diffuse sites of adipose tissue, such as those in the neck region, were not included in this study. On each sampling day, 4 replicate portions of adipose tissue, each derived from between 2 and 10 embryos, depending on the developmental stage, were prepared. The pooling of tissue samples from several embryos was necessary in order to provide sufficient material for the subsequent measurements, particularly at the earlier developmental stages. The intact yolk complexes (i.e., yolk plus yolk sac membrane) were also excised from the eggs and stored at –20°C for up to 2 wk to await lipid analysis. Brains were also dissected from the embryos at various developmental stages and stored at –20°C for up to 2 wk prior to lipid analysis.

Adipocyte preparation. Each replicate portion of adipose tissue was weighed and then minced with scissors to give small pieces of approximately 2 mm^3. A weighed sub-portion (200–400 mg) obtained by random selection of the small pieces was subjected to chloroform/methanol extraction in order to determine the triacylglycerol content of the depot (see below). A further sub-portion (200–400 mg) was incubated in 3 mL of Dulbecco's Modified Eagle's Medium buffered with 25 mM HEPES and containing bovine serum albumin (1%, wt/vol) and collagenase (1 mg/mL) for 1 h at 37°C in a stoppered polypropylene tube with rapid shaking. All constituents of the digestion medium were obtained from Sigma Chemical Co. (Poole, United Kingdom) The digested tissue was gently filtered through a polyester fine mesh (Boots PLC, Nottingham, United Kingdom), and after centrifugation at 300 × *g* for 4 min the infranatant and pellet were carefully removed from beneath the floating fat cell layer with a syringe. The cells were washed twice with 3 mL of Dulbecco's Modified Eagle's Medium containing HEPES (25 mM) and bovine serum albumin (1% wt/vol) and finally resuspended in 1 mL of the same medium. At all stages of cell preparation prior to microscopic analysis, the cells were maintained in medium at 37°C; polypropylene vessels were used throughout.

Measurement of cell size distribution. Adipocytes were examined microscopically using an Olympus Varox (Olympus Optics, United Kingdom) fitted with a combined Nomarski differential interference contrast/phase contrast/brightfield, 1.4NA, aplanatic, achromatic condenser. Brightfield images were obtained using a long working distance plan X10, 0.55NA objective with an X3.3 eyepiece. Images were obtained using a Panasonic CCD camera (Model number WL-CL700/B) (Optivision, Osset, Yorkshire, United Kingdom) mounted vertically on the microscope. The images were analyzed by Bio Scan OPTIMAS software v3.01 (Datacell, Maidenhead, United Kingdom) running on a 25 MHz 386 DX SVGA computer operating with 4 Mb memory, maths coprocessor present, MSDOS 6.2, MS Windows 3.1 with a 20 Mb permanent swap file on a 200 Mb fast hard disk. The main OPTIMAS program and all macro routines were run from a RAM drive created in memory to increase image handling speed and data processing.

Cell measurements were made using a macro coded C++ program specifically written in house for the acquisition/manipulation of the image and the processing of the data. Details of this program are available on request. The image settings for brightfield microscopy were Contrast 220 and Brightness 200. Images were corrected for background distortions and nonuniformity by division of the same image in a blank field. Irregularities created by interference in the color signal from the camera were removed using a color and motion stabilizer. Intact adipocytes were clearly distinguished from oil droplets or debris, and the program exported the cell diameter data as ASC11 code for statistical analysis. Measurements were performed on between 100 and 150 cells examined at random from each replicate sample of adipocytes, giving a total of 400–600 measurements at each developmental stage.

Lipid analysis. Samples were homogenized in a suitable excess of chloroform/methanol (2:1, vol/vol), lipid extracts were prepared, and the amounts of total lipid present were determined gravimetrically (13). Lipids were separated into their major classes by thin-layer chromatography on silica gel G (Merck, Darmstadt, Germany) in a solvent system of hexane/diethyl ether/formic acid (80:20:1, by vol). Following transmethylation (14), the fatty acid compositions of the relevant lipid classes were determined by gas–liquid chromatography using a capillary column system (Carbowax, 30 m × 0.25 mm, film thickness 0.25μ; Alltech, Carnforth, United

Kingdom) in a CP9001 instrument (Chrompack, Middleburg, The Netherlands). The amounts and relative proportions of the fatty acids present were quantified using an EZ Chrom Chromatography Data System (Scientific Software Inc., San Jose, CA). The identities of the peaks were verified by comparison with the retention times of standard fatty acid methyl esters (Sigma Chemical Co.).

RESULTS

Relationship between yolk lipid transfer and adipose tissue development. The results in Figure 1 illustrate the relationship between the amount of total lipid transferred from the yolk complex and the accumulation of triacylglycerol in the adipose depot during development, using two groups of eggs which differed by 1.7-fold in their initial yolk lipid content. In both cases, a relatively small proportion (approximately 9%) of the initial amount of yolk lipid was utilized during the first 12 d of the 21-d embryonic period, and no detectable deposition of adipose triacylglycerol occurred during this time. By contrast, the rapid transfer of the lipid from the yolk from day 12 onward was accompanied by an intensive deposition of triacylglycerol in the adipose tissue which was maintained up to the time of hatching (Fig. 1, A and B). The amount of triacylglycerol that accumulated in the depot was consistently greater for the experiment using eggs with the larger initial level of yolk lipid. For example, by day 19, the triacylglycerol content of the depot and the amount of lipid transferred from the yolk were, respectively, 1.42- and 1.43-fold greater when development was supported by an initial yolk lipid content of 5.7 g as opposed to 3.4 g. For both groups of eggs, the level of adipose tissue triacylglycerol at each time point between days 12 and 19 inclusively was very highly correlated with the amount of lipid transferred from the yolk by that stage ($r = 0.99$, $y = 0.118x - 61.5$, and $r = 0.98$, $y = 0.119x - 46.7$, for the large and small egg experiments, respectively, where x represents mg lipid transferred from the yolk, and y indicates mg triacylglycerol/depot). The relative proportions of triacylglycerol and phospholipid in the yolk remained essentially constant, at approximately 72 and 20 (wt% of total lipid), respectively, throughout incubation.

Between days 19 and 22 (i.e., the period spanning the hatching process), the previously linear relationship between yolk lipid transfer and adipose tissue development completely disappeared. Whereas a considerable proportion (approximately 30%) of the total yolk lipid transfer occurred during this time, no further increase in the amount of adipose triacylglycerol was observed. For the experiment based on the higher initial yolk lipid content, the amount of triacylglycerol in the depot remained approximately constant over the hatching period. However, when development was based on the lower initial yolk lipid level, a significant ($P < 0.01$) decrease

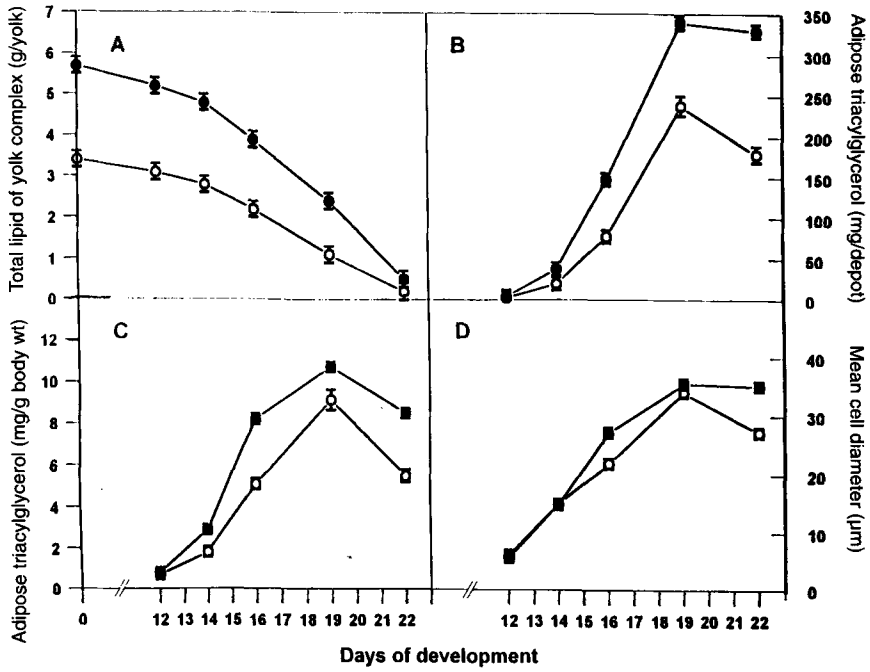


FIG. 1. Relationship between yolk lipid transfer and adipose tissue development. The amount of total lipid present in the yolk complex (A), the amount of adipose tissue triacylglycerol expressed as mg/depot (B) or mg/g body weight (C), and the mean adipocyte diameters (D) are shown during development using two groups of eggs which initially contained either 5.7 ± 0.1 (●) or 3.4 ± 0.1 (○) g total lipid per yolk (means ± SEM, measured on eight eggs from each batch). The results depicted in the figures are the means ± SEM of measurements on four replicate samples at each developmental stage. Day 22 is equivalent to one day after hatching.

of approximately 25% in the amount of adipose triacylglycerol occurred between days 19 and 22.

The size of the embryos and newly-hatched chicks were dependent on the initial egg size and yolk lipid content. For example, the fresh weight (g) of the hatchlings at day 22 were 39 ± 4 and 34 ± 2 (means ± SEM, measured on 4 chicks from each experimental group) for the experiments using the larger and smaller eggs, respectively. However, the lower levels of adipose triacylglycerol in the latter situation were not simply a reflection of the general reduction in embryonic growth since the differences were still apparent when expressed on a body weight basis (Fig. 1C)

Changes in adipocyte size during development. The frequency distributions of adipocyte diameters at different stages of development are depicted by the histograms in Figure 2, in which the values from the 4 replicate samples at each stage are combined. In general, the distributions of cell sizes were relatively symmetrical about the means, approximating to a Gaussian pattern. The changes in the distribution profiles during development were clearly indicative of a continuous increase in cell size within the adipocyte population taking place between days 12 and 19. The pattern of change was very similar irrespective of the initial yolk lipid content. The standard deviation of the sizes within the cell population also increased continuously during development (e.g., from SD = 2.1 μm at day 12 to SD = 8.0 μm at day 19, for the experiment depicted in Fig. 2A), thereby illustrating that adipose tissue growth was accompanied by a greater range of cell sizes. Between days 19 and 22 (i.e., over the hatching period), there was very little change in the adipocyte size profile in the experiment using eggs containing the larger initial amount of yolk lipid (Fig. 2A). In contrast, the experiment based on the lower initial yolk lipid content was characterized by a clear shift in the cell size profile to give a population exhibiting significantly smaller diameters (Fig. 2B).

The developmental changes in mean cell diameter are depicted in Figure 1D, showing the average values of the 4 replicate samples at each stage. The negligible differences between the replicate samples, as indicated by the very low SEM values, reflect the effectiveness of the randomized selection of cells for measurement, and justify combining the values obtained from the replicates to provide a population of sufficient size to produce the histograms shown in Figure 2. Prior to hatching, the mean cell diameters were very similar for the two experiments in spite of utilizing eggs of different initial yolk lipid contents, increasing in both cases from approximately 6 μm at day 12 to approximately 35 μm at day 19; a significant difference ($P < 0.001$) between the two experiments was only observed at day 16. However, whereas the mean adipocyte diameter remained relatively constant over the hatching period following development within the larger eggs, a significant ($P < 0.001$) decrease in cell size over this period, to approximately 27 μm by day 22, was observed following development within the smaller eggs.

Fatty acid composition of adipocyte lipids. Triacylglycerol comprised more than 97% (w/w) of the total lipid from the adipocytes at all stages. The fatty acid composition of adipocyte triacylglycerol for the experiment based on the lower initial yolk lipid content is shown in Table 1. Very similar results were obtained for the experiment based on the higher initial yolk lipid content (not shown). Relatively high proportions of DHA (>6%) were present in the adipocyte triacylglycerol at days 12 and 14, but this proportion decreased considerably by the later developmental stages, so that, in the one-day-old chick, DHA comprised less than 1% of the triacylglycerol fatty acids. The fatty acid composition of the triacylglycerol of the isolated adipocytes was essentially identical to that of the intact tissue at all developmental stages (data not shown). The phospholipid fraction, which at all stages comprised less than 2% (w/w) of the total lipid content of the isolated adipocytes, exhibited proportions of DHA which were very similar to the corresponding triacylglycerol values (Table 2). Thus, the level of DHA in adipocyte phospholipid was 6–7% at days 12 and 14, decreasing to less than 1% in the day-old chick.

Expression of the levels of the individual fatty acids in the triacylglycerol fraction of the intact adipose tissue in terms of mg/adipose depot indicated a unique pattern of change for the

TABLE 1
Fatty Acid Composition of Adipocyte Triacylglycerol

Fatty acid (wt%)[a]	Day of development				
	12	14	16	19	22
16:0	28.0 ± 0.3	26.8 ± 0.1	28.6 ± 0.1	28.6 ± 0.2	27.8 ± 0.6
16:1n-7	2.6 ± 0.1	2.5 ± 0.1	2.7 ± 0.1	2.7 ± 0.1	1.7 ± 0.6
18:0	7.7 ± 0.1	7.7 ± 0.1	7.1 ± 0.1	7.1 ± 0.1	7.9 ± 0.3
18:1n-9	29.6 ± 0.4	31.3 ± 0.3	32.8 ± 0.4	33.9 ± 0.3	34.2 ± 0.8
18:2n-6	20.0 ± 0.1	20.0 ± 0.3	20.9 ± 0.4	22.1 ± 0.3	24.3 ± 0.8
18:3n-3	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.7 ± 0.1	1.6 ± 0.1
20:4n-6	2.2 ± 0.1	2.2 ± 0.1	1.4 ± 0.1	1.1 ± 0.1	0.8 ± 0.1
22:6n-3	6.4 ± 0.3	6.2 ± 0.1	3.7 ± 0.1	1.5 ± 0.1	0.7 ± 0.1

[a]Wt% of total fatty acids. Only fatty acids comprising more than 0.5% are shown. Values are means ± SEM of four replicate adipocyte preparations at each stage, from the experiment based on the initial yolk lipid content of 3.4 g.

TABLE 2
Fatty Acid Composition of Adipocyte Phospholipid[a]

Fatty acid (wt%)[a]	Day of development				
	12	14	16	19	22
16:0	30.7 ± 2.2	23.6 ± 0.1	27.1 ± 1.2	26.0 ± 0.7	28.1 ± 2.5
16:1n-7	0.8 ± 0.8	1.8 ± 0.3	2.2 ± 0.7	2.1 ± 0.2	1.7 ± 0.6
18:0	19.8 ± 0.4	13.0 ± 1.3	12.3 ± 2.5	10.7 ± 0.5	10.9 ± 0.3
18:1n-9	22.3 ± 2.7	27.6 ± 0.8	30.2 ± 1.9	33.2 ± 0.6	33.1 ± 1.1
18:2n-6	13.8 ± 1.3	18.8 ± 0.7	18.5 ± 1.4	20.6 ± 0.2	19.8 ± 2.6
18:3n-3	0.5 ± 0.4	1.0 ± 0.1	1.1 ± 0.1	1.5 ± 0.1	1.1 ± 0.1
20:4n-6	4.3 ± 1.6	5.5 ± 0.7	4.8 ± 0.9	3.9 ± 0.6	3.4 ± 1.3
22:6n-3	6.2 ± 0.9	7.0 ± 0.2	2.5 ± 0.9	1.6 ± 0.1	0.9 ± 0.3

[a]Wt% of total fatty acids. Only fatty acids comprising more than 0.5% are shown. Values are means ± SEM of four replicate adipocyte preparations at each stage, from the experiment based on the initial yolk lipid content of 3.4 g.

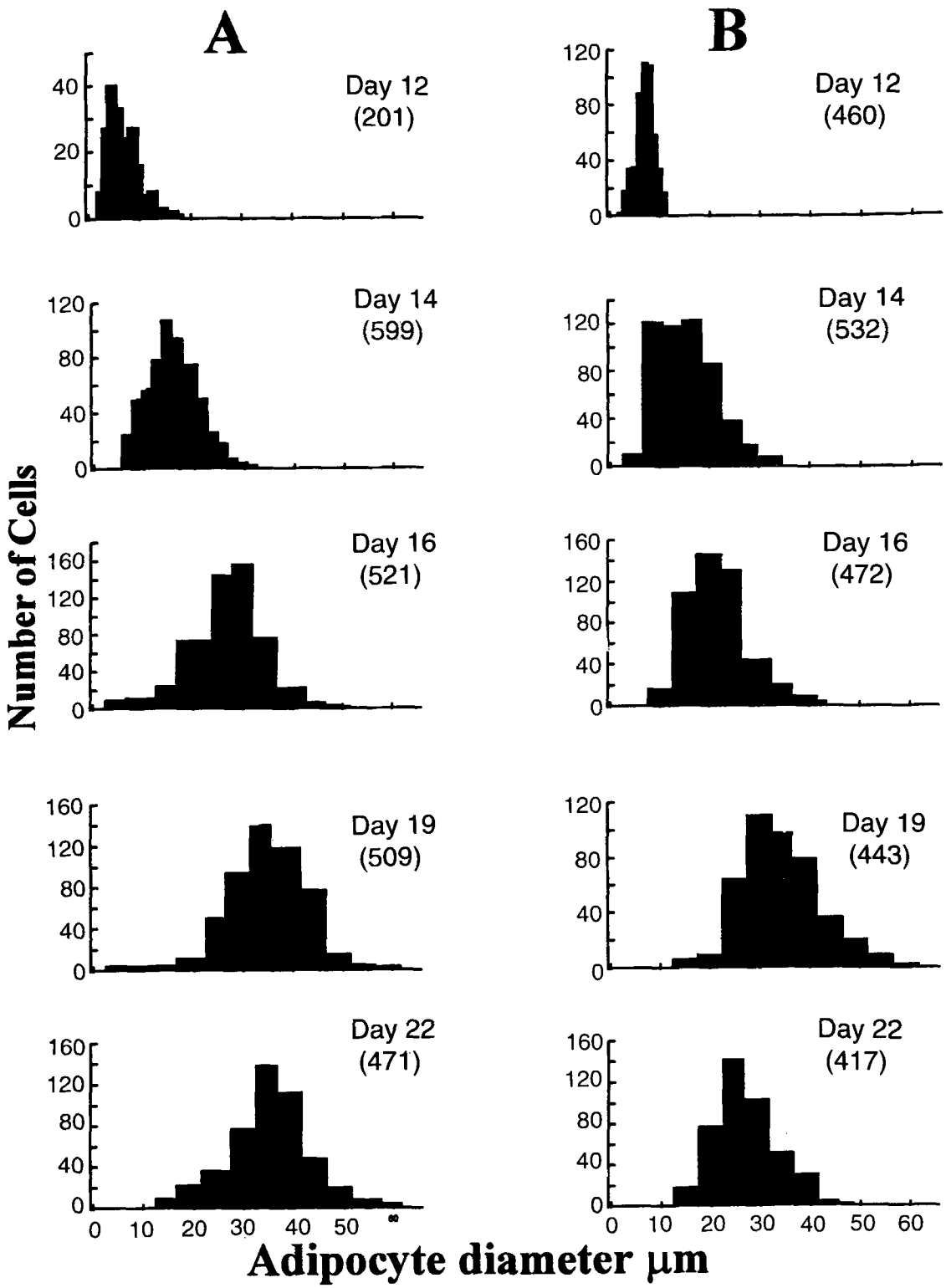


FIG. 2. Frequency distributions of adipocyte diameters during development. The histograms represent the frequency distributions of the adipocyte diameters (μm) in which the measurements from the four replicate samples at each stage are combined. Panels A and B refer to the experiments based on initial mean yolk lipid contents of 5.7 and 3.4 g, respectively. The numbers of cells examined are indicated in the parentheses.

amount of DHA in comparison with the profile for the other major fatty acids (Fig. 3). For example, the amounts of 18:1n-9 (Fig. 3A) and 16:0 (Fig. 3B) in the triacylglycerol fraction of the adipose depot increased continually from day 12 to 19 and thereafter either remained approximately constant (experiment using eggs initially containing 5.7 g yolk

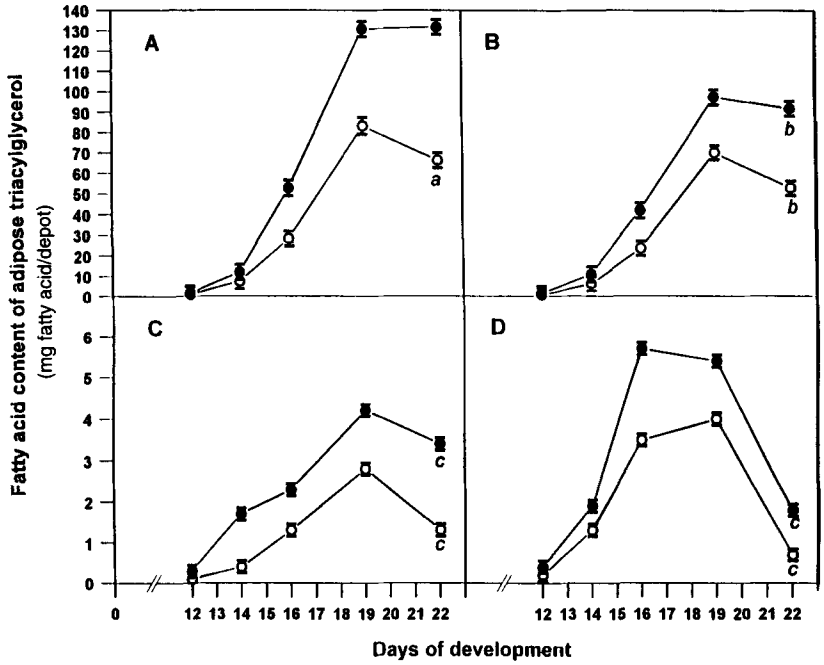


FIG. 3. Amounts (mg fatty acid/depot) of 18:1n-9 (panel A), 16:0 (panel B), 20:4n-6 (panel C), and 22:6n-3 (panel D) in adipose tissue triacylglycerol. The means ± SEM of the four replicate samples at each developmental stage are shown for the experiments based on initial mean yolk lipid contents of 5.7 (●) and 3.4 (○) g. Comparison between day 22 and day 19: $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.

lipid) or decreased slightly (experiment using eggs initially containing 3.4 g yolk lipid) over the hatching period. In contrast, the amount of DHA (Fig. 3D) remained approximately constant between days 16 and 19, but then decreased dramatically over the hatching period. Thus, whereas the amount of 18:1n-9 between days 19 and 22 decreased by 0 and 20% in the two experiments, the corresponding decreases in the levels of DHA were 67 and 83%. Prior to day 19, the changes in the amount of arachidonic acid (Fig. 3C) followed a similar profile to that exhibited by 18:1n-9 and 16:0. However, over the hatching period, the amount of arachidonic acid also decreased by a greater extent (19 and 54% in the two experiments) than the changes observed for 18:1n-9 and 16:0. The amounts of 18:0, 18:2n-6, and 18:3 n-3 are not shown in Figure 4, but the pattern of change of these fatty acids followed very similar profiles to those exhibited by 18:1n-9 and 16:0.

Accumulation of DHA in the developing brain. The amount of DHA present as a component of the phospholipid fraction of the brain was determined at various stages of development in the experiments using the two batches of eggs which differed by 1.7-fold in their initial yolk lipid contents. In both experiments, the DHA content of the brain increased continuously during the period between day 12 of incubation and day 1 after hatching (Fig. 4); in both cases, the scale of the increase in brain DHA over this period was 5- to 6-fold. In spite of the difference in the initial amount of yolk lipid and the 1.4-fold difference in the initial yolk lipid DHA content between the two experiments (see the Materials and Methods section), the levels of DHA in brain phospholipid were very similar in the two cases. For example, at day 19 and day 22, there were no significant differences in brain DHA content between the two experiments.

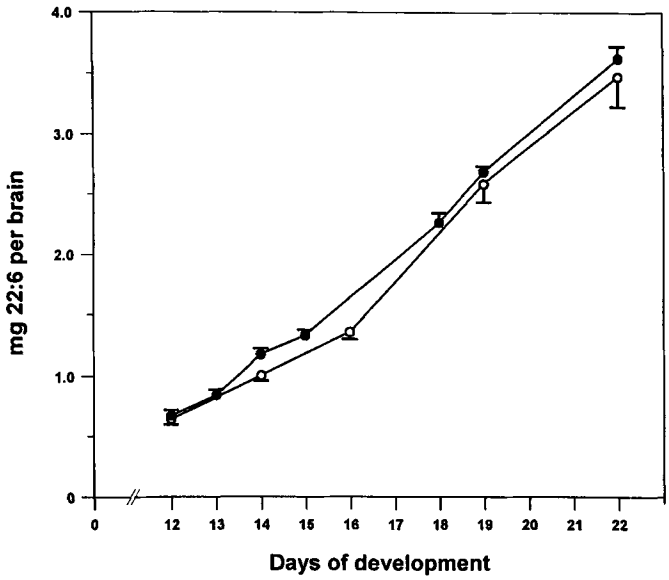


FIG. 4. Accumulation of docosahexaenoic acid (DHA) in brain phospholipid. The amounts of DHA present in the phospholipid of the embryonic brains (mg DHA/brain) during incubation of eggs with initial yolk lipid contents of 5.7 (●) and 3.4 (○) g. Results are the means ± SEM of four brains at each developmental stage.

DISCUSSION

Prior to about day 12 of the chicken's 21-d embryonic period, the rate of transfer of lipid from the yolk to the embryo is relatively low, and subcutaneous adipose tissue cannot be detected (Fig. 1). Thus it is likely that the lipid components which are transferred during this early phase of development are almost entirely utilized for energy purposes or incorporated into the cell membranes of the growing tissues. However, the period around day 12 marks a phase of major metabolic change in this system, characterized by a transition to a state of very intensive transfer of lipid from the yolk to the embryonic tissues (1). The development of subcutaneous adipose tissue is one consequence of this transition, and triacylglycerol accumulates in the tissue at a high rate from day 12 until hatching as indicated in Figure 1B; this is consistent with previous studies (3,6).

Inspection of the data presented in Figure 1 indicates that the amount of triacylglycerol deposited in the adipose tissue between days 12 and 19 is highly correlated with the amount of lipid transferred from the yolk. Furthermore, this linear relationship applies not only to the different stages of development within an experiment, but also to comparisons between batches of eggs containing different initial amounts of yolk lipid. Thus, development within larger eggs, supported by a greater initial amount of yolk lipid, results in the accumulation of increased levels of triacylglycerol in adipose tissue, whether expressed on the basis of mg triacylglycerol per depot or per g body weight (Fig. 1, B and C). Since neither the *de novo* synthesis of lipid from carbohydrate (7), nor the mobilization of lipid from the adipose stores (8), apparently occurs to any significant extent during the period between days 12 and 19, it is likely that the supply of lipid from the yolk in the form of plasma lipoproteins, and the capacity of the tissue to utilize this lipid source mediated by the expression of lipoprotein lipase activity (3) are the key determinants of the rate of adipose tissue growth, during this period. Thus, assuming the level of lipoprotein lipase activity is not limiting, it may be surmised that the growth of the adipose depot will be a function of the amount of lipid transferred from the yolk. The results of the present study (Fig. 1, A and B) are clearly consistent with such a view.

The hatching period marks another major metabolic transition (1) which, in the case of adipose tissue, is typified by a complete breakdown of the previously linear relationship between lipid transfer from the yolk and the accumulation of triacylglycerol in the tissue. Although the intensity of yolk lipid transfer is maintained over this time, with some 30% of the total transfer occurring between days 19 and 22, the growth of the tissue was found to cease completely during this period, and in the case of the experiment based on the lower initial yolk lipid level, the amount of adipose triacylglycerol significantly decreased (Fig. 1). These changes may reflect a combination of a reduced ability of the tissue to obtain lipid from plasma sources due to the reduction in adipose tissue lipoprotein lipase activity which occurs at this time (3), together with an increase in the sensitivity of the tissue to lipolytic hormones (8).

The chick embryo system provides an opportunity to study the characteristics of adipose development from the time of the initial formation of the tissue. A major feature of this process, as shown in Figures 1D and 2, is the marked increase in adipocyte size during the period of rapid lipid deposition. Thus, the mean cell diameter was found to increase from approximately 6 μm at day 12 to about 35 μm by day 19 (Fig. 1D). The adipocyte size frequencies shown in Figure 2 approximate to a Gaussian distribution about the mean value, with no indication of any bimodal distribution of cell sizes which have been associated with increased fat deposition in cattle and pigs (15). Although the use of eggs of different sizes revealed that the triacylglycerol content of the adipose tissue was highly dependent on the initial yolk lipid content (Fig. 1, A and B), the mean adipocyte diameter and cell size distribution from days 12 to 19 were not markedly affected by differences in the initial level of yolk lipid (Figs. 1B and 2). However, small differences in adipocyte diameter can reflect much larger differences in cellular lipid content since cell volume is proportional to the cube of the diameter. Also, the changes in adipocyte number during development were not measured in this study, and so the effects of the initial yolk lipid levels on adipose tissue hyperplasia remain to be evaluated. It should also be noted that the demarcation between adipocytes and preadipocytes is essentially one of methodology, i.e., defined by the accumulation of sufficient lipid within the cell to enable isolation by flotation. It is possible that in the period under study, characterized by the initial genesis and rapid development of the tissue, a significant proportion of tissue lipid may be present in preadipocytes at various stages of differentiation and therefore excluded from the cell size measurements. There is evidence that differential proliferation rates of preadipocytes are associated with differences in fatness between lines of chickens (16).

The triacylglycerol fraction of the isolated adipocytes contained relatively high proportions of DHA (approximately 6%) at days 12 and 14, but with significantly lower proportions at the later stages (Table 1); this is consistent with previous results obtained from analysis of the intact adipose tissue (3). In addition, the results in Table 2 indicate that the adipocyte phospholipids, although quantitatively a minor component and generally displaying a distinct fatty acid composition, contained proportions of DHA which were very similar to those of the corresponding triacylglycerol fraction. Thus the decrease in the proportion of DHA in the triacylglycerol fraction from day 14 onward was paralleled by similar changes in the composition of the adipocyte phospholipid. Previous studies from our laboratory have revealed that the transfer of DHA from the yolk lipids to the embryonic tissues displays a range of unique features not shown by the other major fatty acids. For example, DHA-containing phospholipid species are preferentially taken up from the yolk by the yolk sac membrane at around day 12 (1,12). Moreover, within the yolk sac membrane, DHA is apparently transferred from the yolk phospho-

lipid to triacylglycerol during the assembly of lipoprotein particles for release into the circulation (3,12). The high levels of lipoprotein lipase activity in the adipose tissue (3) of the chick embryo and the fact that lipoprotein triacylglycerol is the preferred substrate for this enzyme (17) are therefore consistent with the uptake of significant levels of DHA by the adipocytes during the period from days 12 to 14.

Expression of the levels of the different fatty acyl components of adipose triacylglycerol in terms of the amount (mg) of each fatty acid per intact depot (Fig. 3) reveals an additional unique feature relating to DHA transfer in the embryo. The amounts of the major C_{16} and C_{18} fatty acids in the tissue increased continuously from day 12 to day 19 and then remained relatively constant or decreased slightly over the hatching period, i.e., parallel to the changes in triacylglycerol content (Fig. 3, A and B). In contrast, the amount of DHA remained relatively constant in the tissue between days 16 and 19 and then decreased precipitously over the hatching period (Fig. 3D). The amount of arachidonic acid in the depot also decreased significantly during hatching, but to a lesser extent than observed for DHA (Fig. 3C).

A possible explanation for these results may be that DHA is preferentially mobilized from the tissue. This may apply not only to the hatching phase but also to the preceding period between days 16 and 19 where the relatively constant levels of DHA in the tissue could reflect a balance between uptake from plasma lipoproteins and mobilization from the adipose triacylglycerol. The existence of such a process would be of considerable relevance to the delineation of the route by which DHA supply is maintained to the brain and retina. The form in which DHA is delivered from the plasma to the developing neural tissues is not known but, as a result of studies on mammalian systems, a range of possibilities has been suggested including the nonesterified fatty acid (18,19), lysophospholipid (20), lipoprotein phospholipid secreted by the liver (21,22), and distinct plasma phospholipid species (23,24). The absence of lipoprotein lipase activity in the brain of the chick embryo (3) precludes the possibility of direct uptake into this tissue following the localized hydrolysis of lipoprotein triacylglycerol. However, the possibility that DHA may be preferentially mobilized from adipose tissue to release the free fatty acid form into the plasma would be consistent with the evidence of Dhopeshwarkar and Mead (18) and Anderson and Connor (19) that polyunsaturated fatty acids are delivered to the brain as the nonesterified form in the plasma.

The period encompassed by the development of subcutaneous adipose tissue in the embryo is also characterized by a marked accumulation of DHA in the phospholipid of the brain (Fig. 4). Although this observation is compatible with a role for adipose tissue in the supply of DHA to the brain, further studies are required to provide a direct test of this concept. It is, however, clear that the amounts of DHA mobilized from the adipose depot between days 19 and 22 are more than adequate to supply the demands of the brain for this fatty acid over the hatching period (Fig. 3D cf Fig. 4).

It is of interest that several studies on human development have implied a role for adipose tissue in the supply of DHA to the neural tissues. For example, it is recognized that maternal adipose stores are extensively mobilized during the second half of pregnancy, providing a supply of polyunsaturated fatty acids to the fetus (25). In addition, the results of Farquarson *et al.* (26) indicate that DHA is selectively depleted from the adipose tissue of the human infant during the first 20 wk of neonatal life. Further studies are needed to assess whether the evidence from the present investigation, suggesting that DHA is preferentially mobilized from the adipose tissue of the chick embryo, can provide insights into the mechanism by which polyunsaturated fatty acids are delivered to the neural tissues during mammalian development.

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Application of 2-Hydroxypropyl-β-Cyclodextrin in the Assay of Acyl-CoA:Cholesterol Acyltransferase and Neutral and Acid Cholesterol Ester Hydrolases

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ABSTRACT: The utility of 2-hydroxypropyl-β-cyclodextrin for increasing the sensitivity of assays for the microsomal acyl-CoA:cholesterol acyltransferase, and the acid lysosomal and the neutral microsomal and cytosolic cholesterol ester hydrolase activity was studied in rat hepatocytes. Enzyme assays, at optimal concentrations of cyclodextrin, were validated by assessing: (i) linearity of product formation with incubation time and protein amount, and saturation with substrate, and (ii) the effect of treatments of cells or of subcellular fractions on enzyme activities. Delivery of cholesterol dissolved in 2-hydroxypropyl-β-cyclodextrin to the acyl-CoA:cholesterol acyltransferase assay mixture raised the enzyme activity more than 8-fold and was twice that measured when cholesterol was added in Triton WR-1339. 2-Hydroxypropyl-β-cyclodextrin itself was partially effective, apparently by making endogenous cholesterol more accesible to the enzyme. Inclusion of 2-hydroxypropyl-β-cyclodextrin in cholesterol ester hydrolase assays using standard micellar substrates doubled the activity estimated in lysosome and microsome preparations and enhanced the cytosolic cholesterol esterase activity by about 50%. Differences in the catalytic activity of acyl-CoA:cholesterol acyltransferase and cholesterol ester hydrolases caused by treatment of hepatocytes with compound 58-035 or 25-hydroxycholesterol, or of subcellular fractions with NaF, were maintained when enzymes were assayed with cyclodextrin. The results indicate that 2-hydroxypropyl-β-cyclodextrin is a suitable vehicle for delivering cholesterol to acyl-CoA:cholesterol acyltransferase and enhances the sensitivity of standard assays of the enzymes governing the intrahepatic hydrolysis of cholesteryl esters.

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The substituted glucopyranose ring structure, 2-hydroxypropyl-β-cyclodextrin (HPBCD), shows a remarkable ability to form aqueous-soluble inclusion complexes with a variety of hydrophobic drugs due to the lipophilic cavity of its seven glucopyranose units (1–3). In particular, HPBCD has been used to prepare stable aqueous solutions of cholesterol, bile acids, and other sterols for both cell culture and *in vivo* studies (4). This has been extended to its use in the assay of cholesterol 7α-hydroxylase and in the 7α-hydroxylation of 27-hydroxycholesterol in microsomal preparations from hamster liver and HepG2 cells (5), and in the synthesis of 27-hydroxycholesterol in rat liver mitochondria (6). The convenience offered by these methods prompted us to investigate the utility of HPBCD in the assay of the intracellular enzymes involved in maintaining the balance of cholesterol between its free and esterified forms within the liver.

The esterification of cholesterol is catalyzed by the enzyme acyl-CoA:cholesterol acyltransferase (ACAT), which is a membrane-bound enzyme localized in the RNA-rich endoplasmic reticulum of the cell (7,8). Standard methods for the radioactive assay of ACAT are based on the incorporation of [1-^{14}C]oleoyl CoA into cholesteryl esters using as substrate the microsomal endogenous cholesterol, which is suboptimal, or a saturating cholesterol concentration. In the latter case, cholesterol is added normally in liposomes (8) or as an aqueous dispersion in Triton WR-1339 (Sigma, St. Louis, MO) (9). The hydrolysis of cholesteryl esters is brought about by the catalytic action of at least three cholesterol ester hydrolases (CEH). The acid lysosomal CEH (lCEH) breaks down cholesteryl esters derived from lipoproteins internalized into cells by receptor-mediated endocytosis, whereas the two neutral forms of CEH activity in microsomes (mCEH) and cytosol (cCEH) catalyze the release of free cholesterol from stores of cholesteryl esters present in cytoplasmic lipid droplets. CEH are commonly measured in terms of the release of labelled oleic acid from a cholesterol [1-^{14}C]oleate micellar preparation, which gives estimates of activity higher than when cholesteryl oleate is suspended in ethanol or acetone. The micellar preparation described by Brecher *et al.* (10) is the most frequently used in the CEH assay and that reported by Hajjar *et al.* (11) in the two neutral CEH assays. Evidence has been obtained in the present work indicating the usefulness of HPBCD for assaying the activity of the microsomal acyl-CoA:cholesterol acyltransferase, with or without added cholesterol as substrate, and for enhancing the sensitivity of standard assays of the acid and the two neutral CEH in subcellular preparations of rat hepatocyte suspensions.

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Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; cCEH, cytosolic CEH; CEH, cholesterol ester hydrolase; DMSO, dimethylsulfoxide; HPBCD, 2-hydroxypropyl-β-cyclodextrin; lCEH, lysosomal CEH; mCEH, microsomal CEH.

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MATERIALS AND METHODS

Chemicals. Cholesteryl [1-^{14}C]oleate (59.5 mCi/mmol), [1-^{14}C]oleic acid (57 mCi/mmol), and [1-^{14}C]oleoyl CoA (56 mCi/mmol) were from The Radiochemical Centre (Amersham, United Kingdom). Cholesteryl [^{3}H]oleate (47 Ci/mmol) was from Du Pont New England Nuclear (Boston, MA). Bovine serum albumin (essentially fatty acid-free), HPBCD, Triton WR-1339, egg lecithin, sodium taurocholate, cholesterol, cholesteryl oleate, 25-hydroxycholesterol, oleic acid, and oleoyl CoA were obtained from Sigma.

Isolation of hepatocytes and sampling for enzyme activities. Female Sprague-Dawley rats (180–200 g), housed in temperature- and light-controlled rooms and maintained on a standard diet *ad libitum* with free access to water, were used for the experiments. Hepatocytes, isolated by collagenase digestion as previously described (12), were suspended in Krebs-Henseleit solution, pH 7.4, containing 2.5 mM $CaCl_2$, 10 mM glucose, and 2.5% bovine serum albumin, to give a cell density of 6–7 $\times$ 10^6 cells/mL. Hepatocytes were maintained in suspension by horizontal shaking, incubated for 30 min prior to addition of the desired compound, and continuously gassed with O_2 /CO_2 (19:1). At the end of the incubation, cells were pelleted and washed twice in ice-cold 20 mM Tris-HCl buffer, pH 7.4, containing 250 mM sucrose, and then centrifuged. The cell pellets were homogenized in 4 vol of the above buffer containing 2 mM EDTA, 10 μM leupeptin, 1 mM benzamidine, and 0.5 mM dithiothreitol, and submitted to differential centrifugation as in Refs. 13 and 14. The lysosome-enriched fraction was resuspended in 250 mM sucrose solution at a protein concentration of 1 mg/mL and microsomes in 20 mM Tris-HCl buffer pH 7.4 containing 250 mM sucrose at a protein concentration of 5 mg/mL. Frozen preparations retained full enzyme activity for at least two weeks.

Assay of ACAT. After the studies that revealed the usefulness of HPBCD, the standard assay mixture contained, in a final volume of 250 μL, 200 μg protein, 80 μM of fatty acid-free bovine serum albumin, 2 mM glutathione, 250 μM cholesterol, 6 mM HPBCD, and 120 μM [1-^{14}C]oleoyl CoA (1000 dpm/nmol) in 100 mM potassium phosphate buffer, pH 7.4. Cholesterol was added as a solution in 10 μL of 45% HPBCD in water, which was prepared as described by De Caprio *et al.* (4). All components except oleoyl CoA were preincubated for 10 min; the reaction was initiated by the addition of oleoyl CoA and was run for 3 min. Enzyme blanks were run in parallel. The reaction was stopped with 5 mL chloroform/methanol (2:1, vol/vol) and cholesterol [^{3}H]oleate (30,000 dpm) was added to each tube to assess recovery. After separation into two layers by the addition of 1.25 mL of 0.88% (wt/vol) KCl, the chloroform layer was removed and dried. The lipids were separated by thin-layer chromatography using *n*-heptane/diisopropyl ether/acetic acid (70:30:2, by vol). Radioactive bands corresponding to cholesteryl oleate were delimited (Multitracemaster analyzer; Berthold, Wildbad, Germany), scraped into vials, and counted for radioactivity by liquid scintillation spectrometry. ACAT activity was expressed as pmol cholesterol esterified per min (1 unit) per mg of protein at 37°C.

Assay of lysosomal, microsomal, and cytosolic CEH activity. CEH activity was determined in terms of release of labelled oleic acid from a cholesteryl [1-^{14}C]oleate micellar substrate. The optimal assay conditions were as follows. For the acid CEH assay, the reaction mixture contained in a final volume of 200 μL, 15 μg of protein, 250 μM of cholesteryl [1-^{14}C]oleate (7000 dpm/nmol), and 15 mM HPBCD in 10 mM acetic acid/Na acetate buffer, pH 4.6. Substrate was prepared basically according to Brecher *et al.* (10) with double concentrations of cholesteryl oleate and lecithin. All components except cholesteryl oleate were preincubated for 10 min; the reaction was initiated by the addition of cholesteryl [1-^{14}C]oleate and was run for 4 min. For the CEH assay, the reaction mixture contained in a final volume of 200 μL, 100 μg of protein, 15 mM HPBCD, 100 μM of cholesteryl [1-^{14}C]oleate (7000 dpm/nmol) incorporated in mixed micelles of egg lecithin and sodium taurocholate (11), and 100 mM imidazole-HCl buffer, pH 6.8. All components except the substrate were preincubated for 10 min; the reaction was initiated by the addition of cholesteryl [1-^{14}C]oleate and was run for 45 min. CEH activity in the cytosol was measured as described for the microsomal form of CEH except that 200 μg of protein, 250 μM of cholesteryl [1-^{14}C]oleate, and 5 mM HPBCD in a final volume of 300 μL solution containing 100 mM imidazole-HCl buffer, pH 6.8, were used, and that the reaction was run for 30 min. In all CEH assays, protein blanks were run in parellel. The released oleic acid was separated from the nonhydrolyzed cholesteryl oleate by adding 5 mL of *n*-heptane/chloroform/methanol/oleic acid (1:1.25:1.4:10^{-4}, by vol) and 1 mL of 50 mM NaOH, H_3BO_3, Na_2CO_3 buffer, pH 11.5. The mixture was shaken and centrifuged to separate the organic layer and the aqueous phase. Aliquots of 1.5 mL of the upper phase were counted for radioactivity. The average efficiency (87%) of extraction of released oleic acid was estimated by running assays using micelles prepared in the same way as the substrate, except that [1-^{14}C]oleic acid was used instead of cholesteryl [1-^{14}C]oleate. The specific activities of all CEH are expressed as units corresponding to hydrolysis of one pmol cholesteryl oleate minute mg protein at 37°C.

Chemical and statistical analysis. Proteins were measured by the dye-binding technique of Bradford (15) using bovine serum albumin as standard. The cholesterol content of microsomal preparations was quantified by high-performance liquid chromatography as described earlier (16), after an exhaustive extraction of the lipids, essentially by the method of Folch *et al.* (17). Statistical significance of results was assessed by the unpaired Student's *t*-test.

RESULTS

The potential utility of HPBCD was first investigated using microsomal, lysosomal, and cytosolic preparations of rat liver cells assayed for ACAT and acid and neutral CEH activities

under the conditions reported to be the optimal in References 9, 10, and 14, respectively. To address this, increasing amounts of HPBCD, as a 45% HPBCD solution (wt/vol) in water (4), were added to enzyme assay mixtures. Cholesteryl oleate exhibited a very low solubility in HPBCD. Following the method referenced above to prepare sterol inclusion complexes, we found that the maximum solubility for cholesteryl oleate at 20°C was 0.9 nmol/mL of a 45% HPBCD solution. A standard CEH assay requires between 20 and 50 nmol cholesteryl oleate for saturation, concentration that one could never reach using the HPBCD solution for presenting cholesteryl oleate to esterases. In the assay of ACAT with added cholesterol, increasing amounts of HPBCD were used to dissolve cholesterol, instead of the detergent Triton WR-1339 (600 μg, as in Ref. 9). Figure 1 shows a similar trend in the effects of HPBCD on ACAT activity whether assayed with endogenous cholesterol, referred to as basal ACAT, or with added exogenous cholesterol. The maximum increases in the cholesterol esterifying activity were seen with 6 or 12 mM cyclodextrin. Activity declined progressively at higher concentrations, so that at 48 mM the activities of both basal ACAT and ACAT dropped below those found in the absence of the solubilizer. However, the magnitude of the effects differ in that basal ACAT activity was increased 3–4-fold by the addition of HPBCD, whereas ACAT activity was doubled by substituting HPBCD for Triton WR-1339 to dissolve cholesterol. A moderate maximum of CEH activity was found with 5 mM HPBCD, and activity diminished progressively above this concentration. As the concentration of HPBCD increased, there was a concomitant increase in the estimated rate of cholesteryl oleate hydrolysis by the lCEH and mCEH, their specific activities being almost tripled by 15–60 mM HPBCD. In the experiments depicted in Figure 1, the reactions were started by adding the substrate after preincubating the enzyme source with HPBCD at 37°C for 10 min, the earliest time point tested. Though not shown, we found that longer preincubation periods resulted in either no further rises or even moderate decreases in activity, i.e., preincubation of microsomes with 250 μM cholesterol in 6 mM HPBCD for 30 min yielded an ACAT activity (2506 ± 127 units/mg protein) lower than that with a 10-min preincubation (3193 ± 30 units/mg protein). In subsequent experiments, HPBCD was included in the assay mixture of ACAT, cCEH, lCEH, and mCEH at 6 mM (10 μL), 5 mM (5 μL), 15 mM (10 μL), and 15 mM (10 μL), respectively, by adding the volume given in parentheses as a 45% HPBCD solution in water.

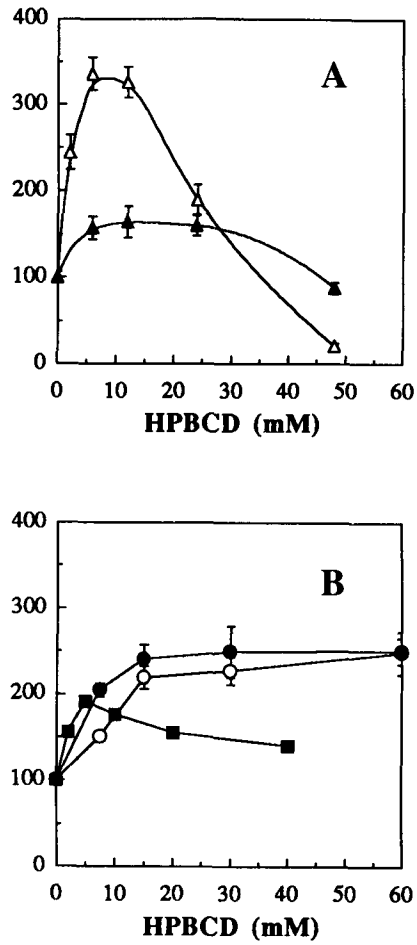


FIG. 1. Effect of increasing concentrations of 2-hydroxypropyl-β-cyclodextrin (HPBCD) on acyl-CoA:cholesterol acyltransferase (ACAT) and the lysosomal, microsomal, and cytosolic cholesterol ester hydrolase (CEH) activity. The activities of (A): ACAT (% of control), with the microsome endogenous cholesterol (27 ± 3 mM) (△) or added 250 μM cholesterol (▲) as substrate, and (B) the lysosomal (○), microsomal (●), and cytosolic (■) CEH (% of control) were measured as described in the Results section in the presence of increasing concentrations of HPBCD. Data are expressed as the percentage of the activity measured without HPBCD that averaged: basal ACAT, 601 ± 49; ACAT, 2031 ± 201; lysosomal CEH: 5813 ± 298; microsomal CEH: 52.5 ± 5.1; and cytosolic CEH: 48.9 ± 6.0 units/mg protein. Values are mean ± SE for three separate experiments. Vertical bars not shown lie within symbols.

The kinetic properties of the enzymes under study were analyzed in the presence or absence of HPBCD. Figure 2 shows the dependence of ACAT activity with the incubation time, protein amount, and exogenous cholesterol. The maximal rate of synthesis of cholesteryl esters was reached by the addition of 6 mM HPBCD preloaded with 250 μM cholesterol, resulting in a neat saturation curve. The rate was about twice that achieved when 250 μM cholesterol was delivered in 600 μg Triton WR-1339 as in Reference 9. The assays contained 120 μM oleoyl CoA and 80 μM bovine serum albumin, which were found to saturate the reaction (data not shown). Under these conditions, the formation of cholesteryl esters was linear with time up to 5 min and with added protein up to 1.2 mg/mL, and the estimated ACAT activity was routinely increased 2-fold by adding cholesterol in HPBCD. Greater percentual increases were found when endogenous cholesterol was esterified. Thus, HPBCD enhanced basal ACAT 3–4-fold. Comparison of the data in Figure 2 indicates that the progress of cholesterol esterification with time and protein amount in microsomes to which only HPBCD was added is close to that observed in microsomes to which cholesterol in Triton WR-1339 had been added.

Figure 3 depicts the dependence of the acid lysosomal, neutral microsomal, and neutral cytosolic CEH activities on

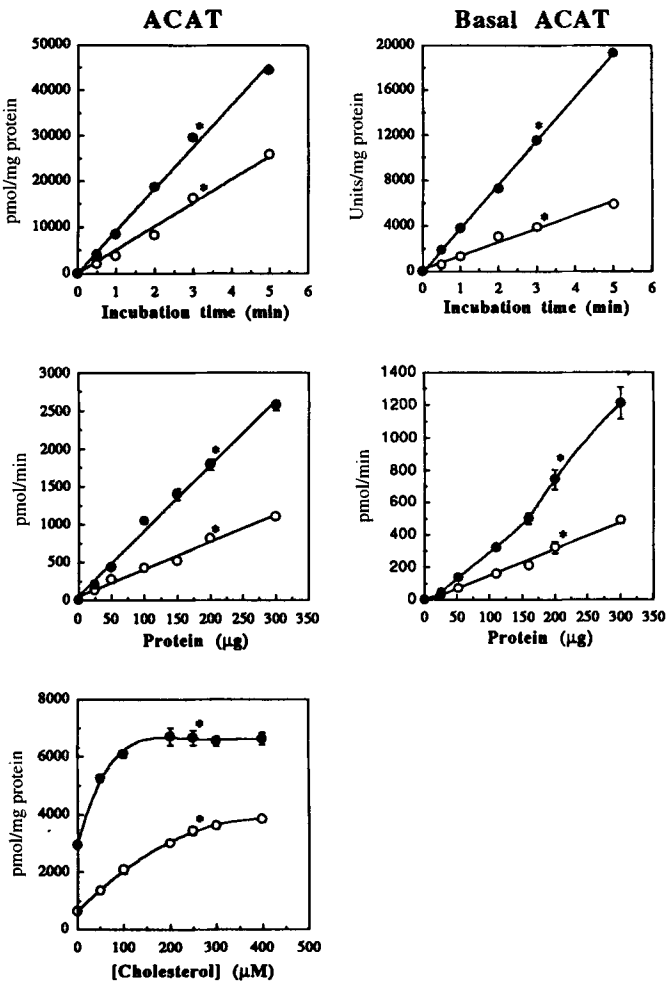


FIG. 2. Dependence of ACAT activity with the incubation time, protein amount, and exogenous cholesterol in the presence or absence of HPBCD. Basal ACAT activity was measured in microsomal preparations with increasing incubation time and protein amount using as substrate endogenous cholesterol (29 ± 4 μM) as described in the Results section in the presence (closed symbols) or absence of 6 mM HPBCD (open symbols). To estimate ACAT under saturation conditions, 250 μM cholesterol was added in 600 μg Triton WR-1339 (Sigma, St. Louis, MO) (open symbols) or 6 mM HPBCD (closed symbols), and these concentrations of Triton WR-1339 and HPBCD were kept constant when ACAT was assayed in the presence of increasing amounts of cholesterol. Values are mean ± SE for three separate experiments. Vertical bars not shown lie within symbols. Asterisks indicate the values used in those experiments where the effect of other variable was tested. Abbreviations as in Figure 1.

incubation time, protein amount, and substrate concentration. Measurements were done with the parameters indicated by asterisks using the micellar substrate described by Brecher *et al.* (10) for the acid and by Hajjar *et al.* (11) for the neutral CEH activities. Addition of optimal concentrations of HPBCD to enzyme assays doubled the lCEH and mCEH activities and enhanced the estimated cCEH activity by about 50%. The reaction rates of the three enzymes showed saturation and were linear with the incubation time and proportional to the protein in the assay. This generalized enhancement of sensitivity enabled us to optimize new conditions for measuring cholesterol esterase activity. They are detailed under the Materials and Methods section, in addition to those to measure ACAT activity. Yet, amounts of enzyme protein much lower than indicated may be used in those studies in which a small amount of enzyme is available.

Preliminary data suggested that HPBCD may also enhance the sensitivity of assays for the mCEH and the lCEH, though not for the cCEH, when cholesteryl oleate is presented as an ethanol solution. We used the optimal assay conditions for the micellar assays, including the corresponding micellar lipids, with the exception that cholesteryl oleate was added in 10 μL ethanol. The presence of 15 mM HPBCD in the assay increased the lCEH activity from 2741 ± 138 to 3403 ± 160 and the mCEH activity from 34.0 ± 3.5 to 65.6 ± 6.8 units/mg/protein (mean ± SE for three experiments). However, 5 mM HPBCD decreased drastically the cCEH activity: from 45.0 ± 3.2 to 16.8 ± 4.8 units/mg protein (mean ± SE for three experiments). The marked ability of cyclodextrin to form inclusion complexes with bile salts (4) may lead to a decreased availability of functional bile salts, which are required to estimate optimally the neutral CEH activity in the cytosol (18). Thus, the addition of HPBCD to a cCEH assay containing bile salts in a nonmicellar form seems unsuitable. Nevertheless, it must be noted that concentrations of HPBCD and assay conditions other than those used here may be required to estimate maximally the acid and neutral CEH activities when cholesteryl oleate is dissolved in ethanol.

As part of the validation of our assays using HPBCD, we evaluated the effect of treatments of cells or subcellular fractions on ACAT and CEH activities. For this purpose, hepatocyte suspensions were treated with compound 58-035 or 25-hydroxycholesterol, which are known to inhibit (19) and to activate (20) ACAT, respectively. As shown in Table 1, 58-035 produced a drastic, dose-dependent inhibition of cholesterol esterification; thus, 0.1 mg/mL 58-035 decreased ACAT activity to 9% and 1 mg/mL to 2.5% of that found in control cells. Differences in catalytic activity persisted when measured in the absence of HPBCD. The synthesis of cholesteryl esters in microsomes from cells exposed to 25-hydroxycholesterol was higher than that in control cells. When ACAT activity was assayed under saturation conditions, the extent of increase was around 36% with both methods. However, the increase in basal ACAT caused by 25-hydroxycholesterol was higher with the HPBCD method (from 39 to 65%). Control cells and cells exposed to 58-035 or 25-hydroxycholesterol exhibited rather constant acid and neutral cholesterol esterase activities regardless of the method used. Further analysis of the potential interaction of HPBCD with enzyme reactions was done with cell-free extracts assayed for enzyme activities in the presence of NaF, a nonspecific inhibitor of esterase activity (Table 2). With HPBCD in the assay, the marked fall in the rate of the three hydrolytic processes was slightly accentuated over that measured in the absence of the solubilizer. With both assay methods, NaF was found to elevate ACAT activity with saturating cholesterol by about 22%, but this was not the case when cholesterol was not added.

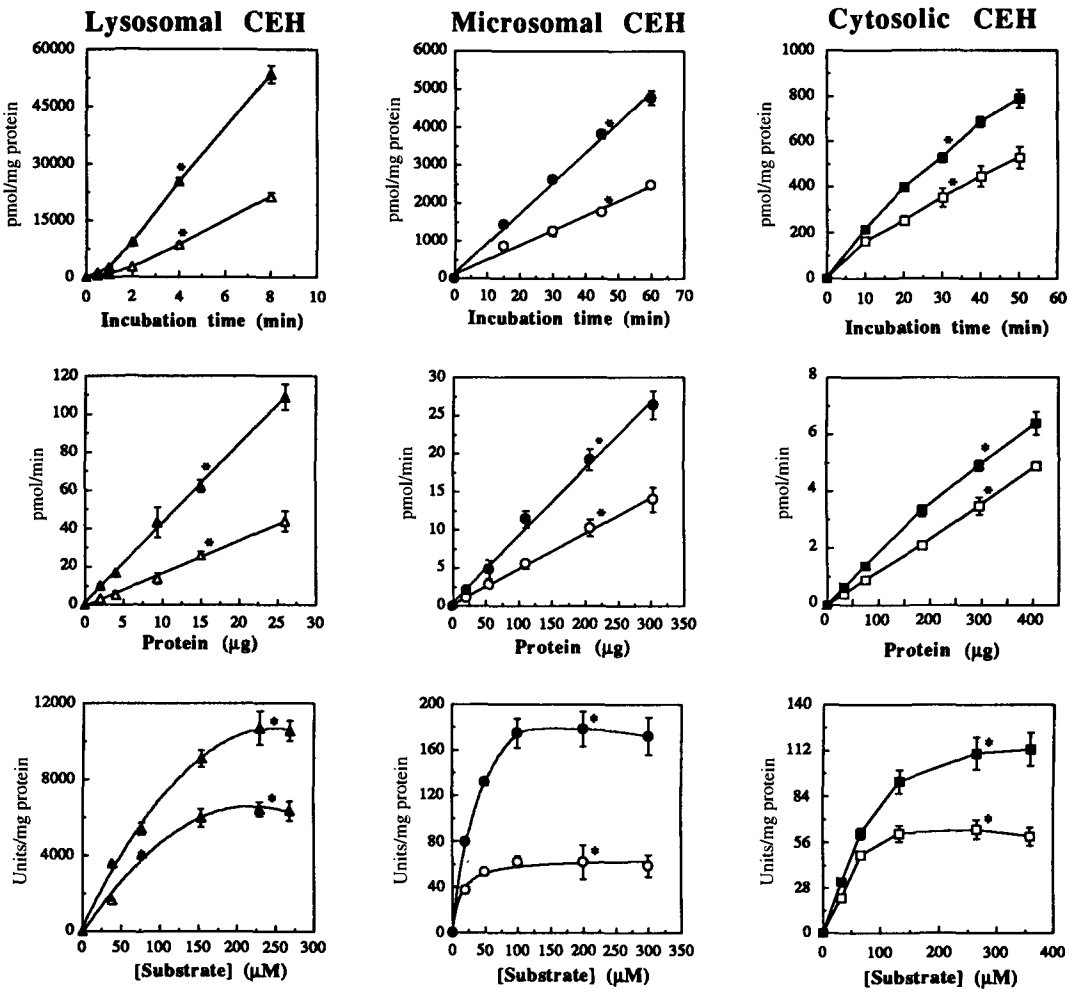


FIG. 3. Effect of HPBCD on the dependence with the incubation time, protein amount, and substrate concentration of the lysosomal, microsomal, and cytosolic CEH activity. CEH activity was measured in lysosomal (▲), microsomal (●), and cytosolic (■) preparations with increasing time of incubation, protein amount, and substrate concentration as described in the Results section with (closed symbols) and without (open symbols) 15, 15, and 5 mM HPBCD, respectively. Values are mean ± SE for three separate experiments. Vertical bars not shown lie within symbols. Asterisks indicate the values used in those experiments where the effect of other variable was tested. Abbreviations as in Figure 1.

TABLE 1
Effect of Treatment of Hepatocyte Suspensions with Compound 58-035 or 25-Hydroxycholesterol on ACAT and the Lysosomal, Microsomal, and Cytosolic CEH Activity[a]

Enzyme (units/mg)	HPBCD	Control (DMSO)	58-035 (1090 μg/mL)	58-035 (1 mg/mL)	Control (ethanol)	25-OH-cholesterol (50 μg/mL)
Basal ACAT	6 mM	2940 ± 104	235 ± 14[c]	69 ± 5[c]	3712 ± 194	6125 ± 186[c]
	—	635 ± 53	59 ± 10[c]	17 ± 4[c]	717 ± 91	997 ± 54[b]
ACAT	6 mM	4590 ± 265	477 ± 25[c]	121 ± 9[c]	6005 ± 245	8174 ± 324[c]
	—	2207 ± 123	208 ± 19[c]	55 ± 6[c]	2420 ± 92	3319 ± 76[c]
lCEH	15 mM	12728 ± 1043	12998 ± 833	11920 ± 1236	11848 ± 589	10650 ± 611
	—	5492 ± 573	4977 ± 181	5218 ± 161	4496 ± 128	4196 ± 245
mCEH	15 mM	161.2 ± 9.3	151.2 ± 13.8	163.8 ± 8.5	200.2 ± 12.5	216.4 ± 11.3
	—	66.8 ± 5.9	64.5 ± 10.1	67.7 ± 7.4	73.1 ± 10.9	79.1 ± 14.2
cCEH	5 mM	118.9 ± 6.3	123.0 ± 9.4	128.4 ± 10.3	91.2 ± 6.5	90.6 ± 7.2
	—	78.3 ± 7.9	77.9 ± 5.6	80.2 ± 8.0	62.8 ± 8.6	61.4 ± 5.1

[a]Liver cells were incubated for 2 h with 0.1 or 1 mg/mL 58-035 added in 10 μL dimethylsulfoxide (DMSO) or 50 μg/mL 25-hydroxycholesterol added in 10 μL ethanol. Controls were incubated with the same volume of the corresponding solvent alone. Cells were homogenized and submitted to subcellular fractionation, and acyl-CoA:cholesterol acyltransferase (ACAT) and cholesterol ester hydrolase (CEH) activities were determined as described in the Materials and Methods section in the presence or absence of 2-hydroxypropyl-β-cyclodextrin (HPBCD). lCEH, lysosomal CEH; mCEH, microsomal CEH; cCEH, cytosolic CEH; 25-Hydroxycholesterol (Sigma, St. Louis, MO); Compound 58-035, Dr. J. Heider (Bando Z. Research Institute, Basel, Switzerland). Comparison between control and treated cells: $^{b}P \leq 0.05$; $^{c}P \leq 0.001$.

TABLE 2
Effect of the Presence of NaF in the Assay Mixture on the Activity of ACAT and the Lysosomal, Microsomal, and Cytosolic CEH[a]

Enzyme (units/mg protein)	HPBCD	Control	50 mM NaF
Basal ACAT	6 mM	3764 ± 236	3499 ± 210
	—	848 ± 65	864 ± 53
ACAT	6 mM	4892 ± 257	5937 ± 298[b]
	—	2220 ± 163	2719 ± 132[b]
lCEH	15 mM	11941 ± 934	1337 ± 108[b]
	—	4155 ± 335	947 ± 79[c]
mCEH	15 mM	197.9 ± 12.9	48.5 ± 7.0[c]
	—	82.4 ± 10.1	28.7 ± 4.6[c]
cCEH	5 mM	88.6 ± 7.3	13.7 ± 1.5[c]
	—	61.7 ± 6.8	14.5 ± 1.8[c]

[a]Control untreated hepatocytes were homogenized and submitted to cell fractionation, and acyl-CoA:cholesterol acyltransferase (ACAT) and cholesterol ester hydrolase (CEH) activities were determined as described in the Materials and Methods section in the presence or absence of 2-hydroxypropyl-β-cyclodestrin (HPBCD); 50 mM NaF was added to assays in 50 μL of the corresponding buffer. Data represent mean ± SE for four experiments done in duplicate. Comparison between control and treated cells: [b]$P \leq 0.05$; [c]$P \leq 0.001$.

DISCUSSION

Endogenous cholesterol is known to be insufficient to saturate ACAT (7–9,20) so that, under nonsaturating conditions, the measurement of activity is substrate limited. When HPBCD is used, all hydrophobic compounds experience a substantial shift into the aqueous phase because of complexation of the molecules with HPBCD (3). Therefore, the fact that the inclusion of cyclodextrin alone in the assay raises ACAT activity about 4-fold suggests that it increases the availability or solubility of cholesterol to the enzyme. Preloading the solubilizer with cholesterol further augmented the rate of cholesterol esterification, to a maximum twice that elicited when ACAT was assayed with cholesterol dissolved in Triton WR-1339. Previous studies indicated that, unlike other vehicles, including detergents, sterols remain in solution within the cavity of the cyclodextrin after subsequent dilutions in aqueous medium (4). It is therefore likely that the aqueous-soluble inclusion complex of cholesterol with HPBCD ensures a more efficient and complete supply of substrate to the site where the enzyme reaction takes place. It has been suggested that regulation of ACAT activity by free cholesterol is due not only to changes in substrate availability, but also to nonsubstrate modulation of enzyme activity (8,21). This is supported by our observation that similar ACAT activities were measured in microsomes with 250 μM cholesterol added in Triton WR-1339 and in microsomes with an HPBCD-enhanced availability of the endogenous substrate, in which cholesterol could reach no more than 22 ± 3 μM, assuming that 80% microsomal free cholesterol is solubilized within 10 min as reported in Reference 5. A part of the activating effect of cyclodextrin might be related to its amphiphilic properties (2). Thus, HPBCD may act as spacer molecules, facilitating the intramembrane movement of cholesterol to the active site of ACAT, or perhaps mimicking an endogenous cholesterol transport system. By this mechanism, high concentrations of HPBCD (i.e., 48 mM) could disturb the endoplasmic reticulum membranes as well as selectively inhibit the activity of the enzymes asymmetrically inserted therein. This is supported by our finding that the enzyme ACAT, whose active site faces the cytoplasm (7), is nearly inactivated by 48 mM HPBCD whereas CEH activity, which resides on the luminal side of the endoplasmic reticulum (22), is not affected. As we found for ACAT, 6 mM HPBCD was also found to be the optimal concentration for assaying the 7α-hydroxylation of cholesterol and 27-hydroxycholesterol in microsomal preparations from hamster liver and HepG2 cells (5). Inasmuch as the enhancement of ACAT activity with endogenous cholesterol by extracellular 25-hydroxycholesterol is higher when the enzyme activity is measured with HPBCD, the availability of the second substrate, 25-hydroxycholesterol (20), for esterification in microsomes also may be increased.

There is considerable evidence that the activities of various CEH are markedly affected by the manner in which the substrate is presented to the enzyme. Cholesteryl esters are insoluble in aqueous media, and the substrate is usually presented either as a solution in ethanol or acetone or suspended in a mixed micelle with bile salts and phospholipids. In consequence, cholesterol esterases act at a lipid–water interface. We use in this work the micellar substrates reported in References 10 and 11 because of the inability of HPBCD to form inclusion complexes with cholesteryl oleate at the concentrations required to saturate CEH assays, and we report here that HPBCD, at optimum concentrations, increases the sensitivity of lysosomal, microsomal, and cytosolic CEH assays. The interpretation of the mode of action of HPBCD is based on the fact that CEH activity is modulated by both qualitative and quantitative changes in the accessibility of the substrate. It is likely that HPBCD activates CEH indirectly, affecting the interfacial quality of the substrate so that the efficiency with which cholesteryl oleate is presented to the enzyme is enhanced. This is supported by the finding that HPBCD increases CEH activity regardless of the mode of presentation of the substrate. In addition, HPBCD may contact the micelle and facilitate the substrate shift from the core toward the surface as well as its interaction with the enzyme. On the other hand, the different degrees and trends of activation caused by HPBCD on the three esterases may be attributable to their different subcellular location. Compared to the soluble enzyme, greater increases of activity were observed in particulate enzymes that reside within the organelle lysosome or on the luminal side of the rough endoplasmic reticulum (22). In both cases the micellar substrate must overcome a physical barrier to reach the enzyme. As mentioned above, the lipophilic properties of HPBCD would lead to its partitioning in cellular membranes, which might facilitate the penetration of the bilayer by cholesteryl oleate. A partial sequestration of micellar bile salts by HPBCD might also be related to the low increase of sensitivity found for the cytosolic CEH assay.

In conclusion, the cyclic oligosaccharide HPBCD at a certain concentration may be used to potentiate substantially the estimation sensitivity of ACAT and the acid and neutral CEH activity. Our assays are useful for testing effects of treatments of cells or of subcellular fractions on the enzymes governing the cholesteryl ester cycle within the hepatocyte.

ACKNOWLEDGMENTS

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Determination of Positional Distribution of Butyryl Groups in Milkfat Triacylglycerols, Triacylglycerol Mixtures, and Isolated Positional Isomers of Triacylglycerols by Gas Chromatography and ^{1}H Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT: Positional isomers (1-butyryl-2*X*-3*Y*-*rac*-glycerol and 2-butyryl-1*X*-3*Y*-*rac*-glycerol; *X,Y* = long-chain acyls) of saturated triacylglycerols (TAG) with 34 and 40 acyl carbons were shown to separate in two chromatographic peaks on immobilized phenyl(65%)methylsilicone column by gas–liquid chromatography, and on reversed-phase ODS-1 column by high-performance liquid chromatography. The analysis of 500-MHz ^{1}H nuclear magnetic resonance (NMR) spectra showed distinct differences between 2-butyryl-1*X*-3*Y*-*rac*-glycerol and 1-butyryl-2*X*-3*Y*-*rac*-glycerol isomers in the resonance signals of methylene and methine protons of glycerol backbone, and carbon-2 methylene of acyl groups, and methyl protons of butyryl group. The ^{1}H NMR spectra of three interesterified mixtures of three monoacid TAG containing saturated butyrate and caproate TAG and unsaturated butyrate TAG showed that triplets of methyl protons of butyryl groups at *sn*-1(3)- and *sn*-2-positions in saturated and unsaturated TAG had similar chemical shifts and that the chemical shift of caproyl methyl protons was different from those of butyryl methyl protons. The positional distribution of butyryl groups in isolated positional isomers of butyrate TAG, interesterified TAG mixtures, and natural and interesterified butteroil can be determined by integration of these signals.

Lipids 31, 331–336 (1996).

The determination of positional distribution of acyl groups between *sn*-1(3) and *sn*-2 positions in the molecular species of triacylglycerols (TAG) or TAG mixtures can be carried out by pancreatic lipase deacylation (1), by Grignard degradation with allyl magnesium bromide (2), and by mass spectrometric methods (3). Low-resolution ^{1}H nuclear magnetic resonance (NMR) spectra with lanthanide chemical shift reagents have been shown to differentiate the positional isomers of dipalmitoyloleoylglycerol (4) and those of unusual monoisovaleroyldipalmitoylglycerol and di-isovaleroylpalmitoylglycerol (5). ^{1}H NMR spectra at 220 MHz with chiral chemical shift reagents have been used to show the absolute configuration of a monobutyryl TAG fraction of hydrogenated butterfat to be 1,2-diacyl-3-butyryl-*sn*-glycerol (6). Differences in the chemical shifts of carbon-2 (7), olefinic carbon (8), carbonyl carbon (9), and n-3 carbons of saturated TAG isomers (10) in the ^{13}C NMR spectra have been used to deduce the position of acyl groups. The acyl distribution and acyl positional distribution in vegetable oils have been examined by quantitative high-resolution ^{13}C NMR (11). Recently, ^{1}H-^{13}C correlated NMR spectroscopy has been used to assist quantitative one-dimensional ^{1}H and ^{13}C evaluation of positional isomers of an interesterified TAG mixture (12). Our aim was to study the possibility to determine the distribution of short-chain acyl groups between primary and secondary positions in TAG of natural fats by high-resolution gas–liquid chromatography (GLC) and high-resolution ^{1}H NMR. To achieve this objective and to reveal the reliability of the determination, interesterified mixtures of TAG of known composition, isolated short-chain acyl TAG isomers, and interesterified and natural butteroil were studied.

MATERIALS AND METHODS

Three equimolar mixtures of three monoacid TAG [tributyrin, trilaurin, tristearin; tricaproin, tripalmitin, tristearin; and tributyrin, tristearin, triolein (Fluka AG, Buchs, Switzerland)] and butteroil were interesterified at 90°C using 1% sodium methoxide as catalyst. The TAG of the reaction mixtures were isolated on silica gel thin-layer chromatography (TLC) plate as described by Christie (13), or on a 5-cm × 2-cm silica gel column, which was eluted with mixture of hexane/diethyl ether (95:5, vol/vol). For the assignment of ^{1}H chemical shifts, 2-butyryl-1,3-distearoylglycerol was synthesized according to the principles reported by Jensen and Pitas (14) as follows: 1,3-distearoylglycerol (Nu-Chek-Prep, Elysian, MN) (40 μmol), butanoyl chloride (60 μmol), and pyridine (60 μmol) in chloroform (0.6 mL) were stirred at 25°C for 7 d.

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Abbreviations: GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; TAG, triacylglycerols; TLC, thin-layer chromatography; TMS, tetramethylsilane.

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TAG of the reaction mixture were isolated by TLC on a silica gel plate (Merck, Darmstadt, Germany) using hexane/diethyl ether/ formic acid 80:20:2 as developing solvent (13).

TAG were analyzed on a Carlo Erba gas chromatograph (Milan, Italy) with an on-column injector, a flame-ionization detector, and a constant pressure/constant flow cp-cf 516 module. The chromatograms were run on a 25-m 0.25-mm i.d. immobilized phenyl(65%)methylsilicone column (Quadrex, New Haven, CT) with 0.1-μm film thickness. Injection was made with a constant carrier gas (hydrogen) pressure as described by Kemppinen and Kalo (15). Immediately after injection, the cp-cf module was changed to constant flow mode (linear velocity 62 cm/s). Temperature program was: After 3 min at 180°C, the column temperature was increased at 10°C/min to 310°C, 0.5°C/min to 320°C, and further 2°C/min to 360°C (hold 4 min).

TAG isomers were isolated by reversed-phase high-performance liquid chromatography (HPLC) using a Hewlett-Packard 1090 series II liquid chromatograph with an effluent splitter (split ratio 1:1) and an evaporative light-scattering detector (Cunow Model DDL 21; Cunow, Cergy Saint-Christophe, France). The detector was operated at 54°C with air pressure of 1.1 bar. The isolation was performed on a 25-cm × 0.46-cm Spherisorb S5ODS1 column (PhaseSep, Deeside, United Kingdom) with 5 μm particle size, 8 nm pore diameter, and 7% carbon coverage using a linear gradient of 1–70% isopropanol in acetonitrile and a flow rate of 1 mL/min (90 min).

^{1}H NMR spectra were recorded in $CDCl_3$ with Varian Unity 500 (11.74T) spectrometer (Palo Alto, CA) and referenced to internal tetramethylsilane (TMS) (0.00).

RESULTS AND DISCUSSION

In gas chromatographic analyses, the polarizable phenylmethylsilicone columns have been proven to separate TAG isomers with different acyl chains in the acyl carbon number range 24–42 due to polarity differences of these isomers (16). When the interesterified equimolar mixture of tributyrin, trilaurin, and tristearin was analyzed on such column, each of the TAG with 28, 34, and 40 acyl carbons eluted in two peaks with ratios 1.84:1, 1.92:1, 1.98:1, respectively (Fig. 1). The ratios were close to the expected random ratio 2:1. We concluded that 2-butyryl-1*X*-3*Y*-*rac*-glycerol (*X,Y* = long-chain acyls) isomers were separated from 1-butyryl-2*X*-3*Y*-*rac*-glycerol isomers. To identify these isomeric TAG, we studied the possibility of isolating them using reversed-phase HPLC column. The ODS-1 column separated TAG with 34 and 40 acyl carbons in two peaks, when a linear gradient of 1–70% isopropanol in acetonitrile was used (Fig. 2). Despite numerous attempts, complete separation of TAG 28 isomers could not be achieved by modification of gradient. Relative to gas chromatographic separation, the TAG isomers eluted in the reverse order, which was expected for the separation on a reversed-phase column. This is consistent with polarity differences of TAG isomers. A *ca.* 6 mg sample of interesterified mixture was subjected to a preparative separation of TAG 34 and TAG 40 isomers on the analytical HPLC column used.

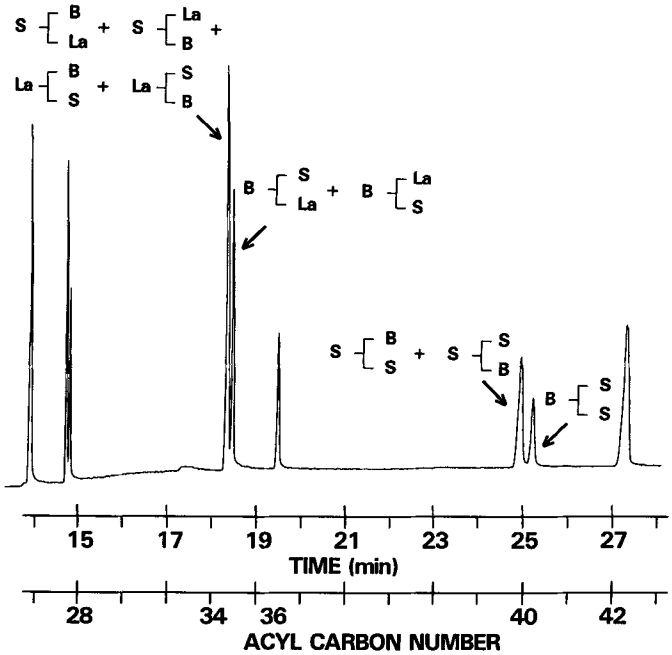


FIG. 1. Separation of triacylglycerols of interesterified mixture of tributyrin, trilaurin, and tristearin on a 25-m 0.25-mm i.d. immobilized phenyl(65%)methylsilicone column with 0.1-μm film thickness using cold on-column injection, and hydrogen as carrier gas (linear velocity 62 cm/s). Abbreviations: B = butyryl, La = lauroyl, and S = stearoyl.

The ^{1}H NMR spectrum of the TAG 40.1 (Fig. 2) collected from the HPLC separation and that of the synthesized 2-butyryl-1,3-distearoylglycerol were identical (Fig. 3, A and B), and both were similar to the spectrum of TAG 34.1 (Table 1). The ^{1}H NMR spectra of TAG 40.2 and TAG 34.2 were almost similar, but they differed markedly from those of TAG 40.1 and TAG 34.1 in the resonance signals of methylene and methine protons of glycerol backbone, the C-2-methylene protons of acyl groups, and the methyl protons of butyryl group (Table 1; and Fig. 3, A, B, and C). The ^{1}H spectrum of the TAG 40.1 (and TAG 34.1) showed AB part of an ABX system of methylene and methine protons of glycerol backbone at δ 4.161 (4.162) ppm and 4.306 (4.307) ppm, and X part at δ 5.28 ppm; and coupling constants J_{AX} 5.9 Hz, J_{BX} 4.2 (4.4) Hz, and J_{AB} 11.9 Hz (Table 1). The ^{1}H spectrum of the TAG 40.2 (and TAG 34.2) showed two overlying ABX systems of methylene and methine protons of glycerol backbone (Table 1; Fig. 3C). The δ-values for A_IB_I methylene protons were 4.160 and 4.305 ppm, and those for $A_{II}B_{II}$ 4.164 and 4.309 (4.308) ppm, δ_X 5.28 ppm and coupling constants J_{AIX} = 5.9 Hz, J_{AIIX} = 6.0 Hz, J_{BIX} = 4.2 (4.3) Hz, J_{BIIX} = 4.4 Hz, and $J_{AIBI} = J_{AIIBII}$ = 11.9 Hz (Table 1). The ^{1}H NMR spectra of the TAG 40.1 and TAG 34.1 showed two overlying triplets of C-2 methylene protons of acyl groups at δ 2.318 and 2.320 ppm (Table 1; Fig. 3, A and B). Three overlying triplets of C-2 methylene protons were at δ-values 2.327, 2.320, and 2.312 ppm in the spectra of TAG 40.2 and TAG 34.2 (Table 1; Fig.

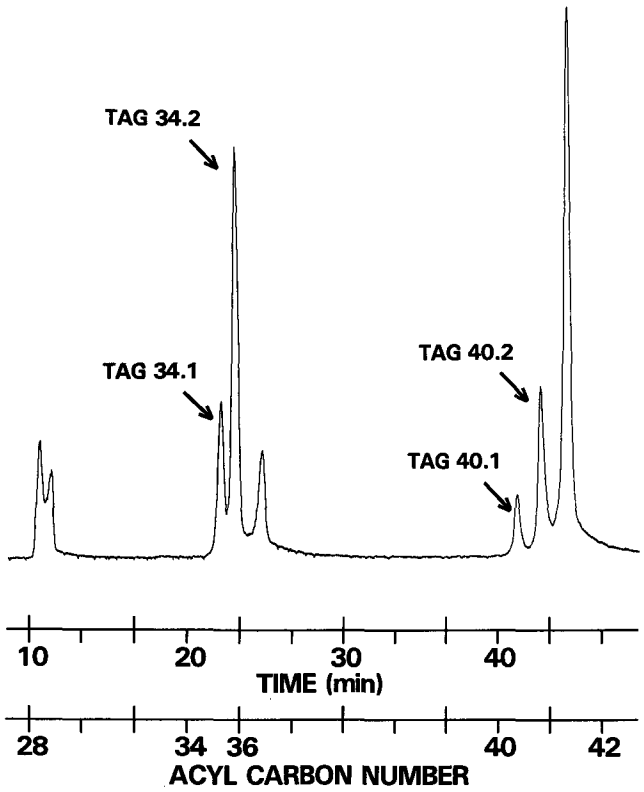


FIG. 2. Reversed-phase high-performance liquid chromatography chromatogram of triacylglycerols (TAG) of interesterified mixture of tributyrin, trilaurin, and tristearin on a 25-cm × 0.46-cm ODS-1 column using linear gradient of 1–70% isopropanol in acetonitrile.

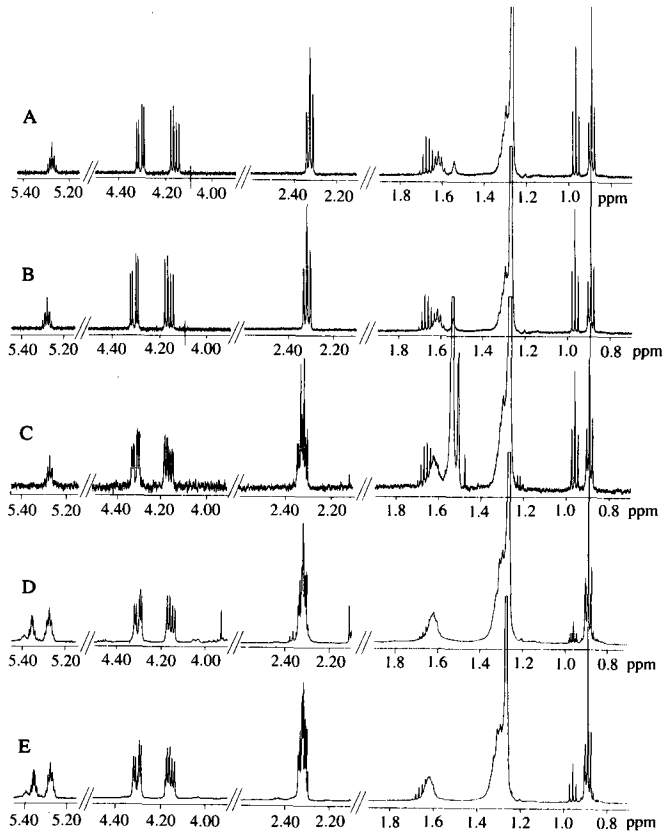


FIG. 3. 500-MHz ^{1}H nuclear magnetic resonance spectra of synthesized 2-butyryl-1,3-distearoylglycerol (A), isolated 2-butyryl-1,3-distearoylglycerol (B), 1-butyryl-2,3-distearoyl-*rac*-glycerol (C), interesterified butteroil (D), and natural butteroil (E). Chemical shifts are referenced to tetramethylsilane (0.00).

3C). The spectra of TAG 40.2 and TAG 34.2 showed that the two methylenes of glycerol backbone, and the C-2 methylenes of acyl groups, are in different environment.

The ^{1}H NMR spectrum of the TAG 40.1 (and TAG 34.1) showed a triplet of methyl protons of 2-butyryl group at δ 0.967 (0.966) ppm, and a triplet of long-chain acyl protons at δ 0.892 ppm (Table 1; Fig. 4, A and B). The δ-value of the triplet of methyl protons of 1(3)-butyryl group of TAG 40.2 (and TAG 34.2) was 0.960 ppm differing from the δ-value of the methyl protons of 2-butyryl group of TAG 40.1 (and TAG 34.1) 0.007 (0.006) ppm (Table 1; and Fig. 4C).

In addition, ^{1}H NMR spectra of all TAG isomers showed a sextet of C-3 methylene of butyryl group within δ 1.6–1.7 ppm partially overlying with signals of C-3 methylenes of long-chain acyls, and an intense signal of methylene protons of long-chain acyl groups at δ 1.266 ppm (Table 1; and Fig. 3, A, B, and C).

The integration of the signals of synthesized 2-butyryl-1,3-distearoylglycerol showed ratios of protons of glycerol methine/glycerol methylene/C-2 methylene of acyl groups/methyl of butyryl group/methyl of stearoyl group 1:3.98:5.73:2.73:5.81, which are consistent with the structure. The spectra of TAG 34.1, TAG 40.2, and TAG 34.2 are consistent with the structures of 1-lauroyl-2-butyryl-3-stearoyl-*rac*-glycerol, 1-butyryl-2,3-distearoyl-*rac*-glycerol, 1-butyryl-2-lauroyl-3-stearoyl-*rac*-glycerol and 1-butyryl-2-stearoyl-3-lauroyl-*rac*-glycerol, respectively.

To reveal the possibility of determining the distribution of butyryl groups between *sn*-1(3)- and *sn*-2-positions in TAG mixtures, the NMR spectra of three interesterified equimolar mixtures of three monoacid TAG were recorded. The ^{1}H NMR spectrum of the interesterified mixture of tributyrin, trilaurin, and tristearin containing *ca.* 63% butyrate TAG showed two triplets at δ 0.957 and δ 0.964 ppm representing resonance signals of methyl protons of butyryl groups at *sn*-1(3)-positions and at *sn*-2-position, respectively (Fig. 4D). The intensity ratio of the most intense signals was 1.96:1, and that of the sum of the triplet signals 1.85:1, which agreed with the ratio of theoretical value of 2:1 and those of the isomers of TAG with 28, 34, and 40 acyl carbons of GLC analysis (1.84:1–1.98:1).

The ^{1}H NMR spectrum of the interesterified mixture of tricaproin, tripalmitin, and tristearin revealed that the signals of methyl protons of caproyl groups in the range 0,907–0,921 ppm do not disturb the determination of positional distribution of butyryl groups (Fig. 4E).

The ^{1}H NMR spectrum of the interesterified mixture of tributyrin, tristearin, and triolein containing saturated, monoene, and diene butyrate TAG showed two triplets at δ 0.960 ppm and at δ 0.966 ppm of methyl protons of butyryl groups

TABLE 1
^{1}H Nuclear Magnetic Resonance Signals: Chemical Shifts (δ, ppm) and Coupling Constants (J, Hz)

	Triacylglycerols			
δ/J	SBS[a] TAG 40.1	*rac*-BSS[b] TAG 40.2	*rac*-LaBS[c] TAG 34.1	*rac*-BLaS[d] *rac*-BSL TAG 34.2
ABX system of glycerol backbone				
δ_{AI}	4.161	4.160	4.162	4.160
δ_{AII}		4.164		4.164
δ_{BI}	4.306	4.305	4.307	4.305
δ_{BII}		4.309		4.308
δ_X	5.28	5.28	5.28	5.28
J_{AIX}	5.9	5.9	5.9	5.9
J_{AIIX}		6.0		6.0
J_{BIX}	4.2	4.2	4.4	4.3
J_{BIIX}		4.4		4.4
J_{AIBI}	11.9	11.9	11.9	11.9
J_{AIIBII}		11.9		11.9
Overlying triplets of C-2 methylene protons	2.318 2.320	2.312 2.320 2.327	2.320	2.312 2.321 2.327
Signals of C-3 methylene of butyryl group and those of long-chain acyl group	1.6–1.7	1.6–1.7	1.6–1.7	1.6–1.7
Signals of methylenes of long-chain acyls	1.266	1.266	1.266	1.266
A triplet of methyl protons of butyryl	0.967	0.960	0.966	0.960
A triplet of methyl protons of long-chain acyls	0.892	0.892	0.892	0.892

[a]Synthesized 2-butyryl-1,3-distearoylglycerol; TAG, triacylglycerol.
[b]1-Butyryl-2,3-distearoyl-*rac*-glycerol.
[c]1-Lauroyl-2-butyryl-3-stearoyl-*rac*-glycerol.
[d]1-Butyryl-2-lauroyl-3-stearoyl-*rac*-glycerol, 1-butyryl-2 stearolyl-3-lauroyl-*rac*-glycerol.

at *sn*-1(3)-positions and at *sn*-2-position, respectively (Fig. 4F). Integration of signals revealed distribution of buturyl groups between *sn*-1(3)- and *sn*-2-position in the ratio 1.77:1 according to the most intense signals and in the ratio 1.74:1 according to the sum of the triplet signals.

The ^{1}H NMR spectra of the three interesterified mixtures of three monoacid TAG revealed that positional distribution of butyryl groups in the mixtures of saturated, monoene, and diene TAG can be determined on the basis of triplets of methyl protons. The ^{1}H NMR spectra of natural and interesterified butteroil were recorded to prove the applicability of the method in analyzing natural fats. The spectrum of interesterified butteroil showed the triplets of methyl protons of butyryl groups at the *sn*-1(3)-position and at *sn*-2-position at δ 0.959 and δ 0.965 ppm, respectively, in the ratio 1.82:1 of most intense signals and in the ratio 1.71:1 of the sum of signals, which agree with the random ratio 2:1 (Fig. 4G). In the spectrum of natural butteroil, only one triplet of methyl protons of buturyl group at δ 0.958 ppm was observed, indicating that butyric acid is entirely esterified with primary hydroxyls (Fig. 4H). This is in accordance with the results of stereospecific analysis using Grignard degradation and enzymatic deacylation (17–19), and ^{1}H NMR spectroscopy with chiral shift reagent of monobutyryl TAG fraction of hydrogenated butterfat (6), which indicated that butyric acid is predominantly esterified at the *sn*-3-position, and the results of regiospecific analysis using ^{13}C NMR spectroscopy, which demonstrated the primary isomeric positioning of butyryl groups (>97%) (7).

The spectra of interesterified mixtures of tributyrin, trilaurin, and tristearin; and tributyrin, tripalmitin, and triolein, showed several overlying ABX systems of methylene and methine protons of glycerol backbone around δ 4.16 ppm (A) and 4.30 ppm (B) and δ 5.28 ppm (X) and the spectrum of interestrified mixture of tricaproin, tripalmitin, and tristearin only one ABX-system. In the spectra of butteroils, the expected overlying ABX systems were poorly resolved (Fig. 3, D and E). On the contrary, to the isolated positional isomers of butyryl TAG, the signals of glycerol backbone protons of TAG mixtures and butteroils cannot been used in identifying the position of butyryl groups. Spectra of all interesterified mixtures and both butteroils showed overlaying triplets of C-

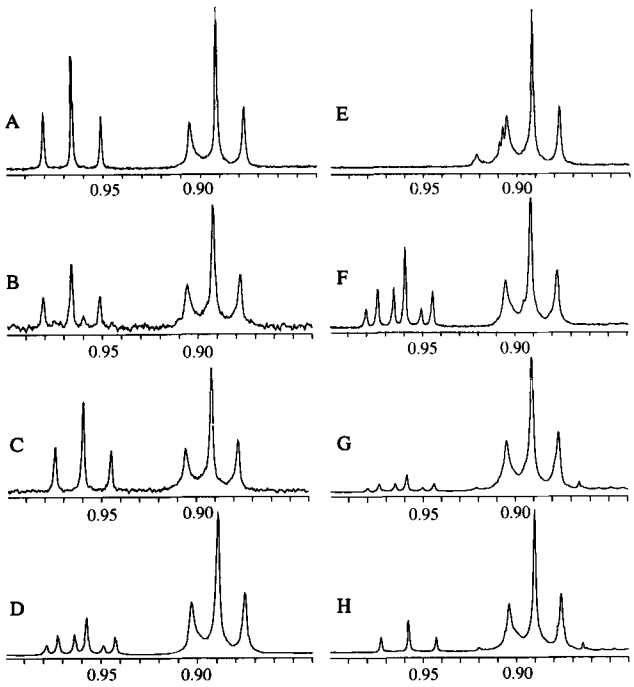


FIG. 4. Resonance signals of the methyl protons of butyryl group of synthesized 2-butyryl-1,3-distearoylglycerol (A), isolated 2-butyryl-1,3-distearoylglycerol (B), 1-butyryl-2,3-distearoyl-*rac*-glycerol (C), interesterified equimolar mixtures of tributyrin, trilaurin, and tristearin (D); tricaproin, tripalmitin, and tristearin (E); tributyrin, tristearin, and triolein (F), interesterified butteroil (G), and natural butteroil (H).

2 methylene protons around δ 2.3 ppm, signals of C-3 methylene protons of butyryl groups around δ 1.65 ppm (except the spectrum of caproyl TAG), signals of C-3 methylene protons of long-chain acyl groups around δ 1.62 ppm and signals of other methylenes of long-chain acyls in the range δ 1.2–1.3 ppm and a triplet of methyl protons of long-chain acyls around δ 0.89 ppm. The spectra of interesterified mixture of tributyrin, tristearin, and triolein, and both butteroils showed signals of olefinic protons at δ 5.35 ppm and allylic methylene protons at δ 2.02 ppm (20).

In the present study, the polarizable phenyl(65%)methylsilicone column showed equal or better separation efficiency of positional isomers of butyrate TAG than reported recently for unspecified short-chain TAG isomers (21). The resolving power of these columns is sufficient to be used in the determination of positional distribution of butyric acid in the simple mixtures of butyrate TAG. It is likely that also positional isomers of other short-chain TAG, at least caproates, can be separated by GLC. Work is in progress to study to which extent GLC can be used in the determination of positional distribution of short-chain TAG in simple mixtures and natural fats. The determination of positional distribution of butyryl groups by ^{1}H NMR spectroscopy can be done without any other treatments than drying of a fat sample and from much smaller sample than by ^{13}C NMR (7). ^{1}H NMR spectra can be easily recorded from a sample below one milligram. The method is thus useful in the structural analysis of molecular species of butyrate TAG isolated by combined chromatographic techniques.

In conclusion, the results clearly demonstrated the resolving power of polarizable phenylmethylsilicone (GLC) column and reversed-phase ODS-1 (HPLC) column in separation 2-butyryl and 1(3)-butyryl isomers of saturated TAG. We showed that the 500-MHz ^{1}H NMR spectra differentiated 2-butyryl and 1(3)-butyryl isomers, and that the determination of positional distribution of butyryl groups in simple mixtures of saturated, monoene, and diene butyrate TAG and in isolated positional isomers of butyrate TAG can be done by GLC or ^{1}H NMR spectroscopy. We showed also the applicability of ^{1}H NMR spectroscopy in determining the positional distribution of butyryl groups between *sn*-1(3)- and *sn*-2-positions in TAG of milkfat by direct measurement of dried milkfat samples.

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Mechanism of Lower Oxidizability of Eicosapentaenoate Than Linoleate in Aqueous Micelles

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ABSTRACT: The aerobic oxidation kinetics of methyl eicosapentaenoate (20:5n-3) and methyl linoleate (18:2n-6) were compared in homogeneous chlorobenzene solution and in Triton X-100 aqueous micelles at 37°C. The rate of disappearance of 20:5n-3 was two times faster than that of 18:2n-6 in chlorobenzene, while the former was five times slower than the latter in aqueous micelles. It was also observed that ΔO_2 = Δ18:2n-6 and ΔO_2 = 2 Δ20:5n-3 in aqueous micelles. In the oxidation of a 1:1 mixture of 20:5n-3 and 18:2n-6 in micelles, the rate of disappearance of 20:5n-3 was 3.6 times faster than that of 18:2n-6, and the rate of total substrate disappearance was reduced by a factor of 5 as compared with 18:2n-6 oxidation. These data suggest that the peroxyl radical derived from 20:5n-3 is more polar than that from 18:2n-6, and the former is likely to diffuse from the core to the micelle surface. This lowers the oxidizability for 20:5n-3 in aqueous micelles by enhancing the termination reaction rate for peroxyl radicals and by reducing the rate of propagation since there may be more 20:5n-3 peroxyl radicals at the surface than in the micelle core.
Lipids 31, 337–340 (1996).

Polyunsaturated fatty acids (PUFA) are particularly susceptible to oxidation since they have chemically labile, bisallylic hydrogen substituents (1–4). Therefore, the oxidizability of PUFA should be dependent on the number of bisallylic positions available for oxidation. This has been confirmed for the oxidation in homogeneous solution (1–4). On the contrary, it has been recently reported that the oxidizability of PUFA in aqueous micelles decreased with an increasing number of bisallylic sites available for oxidation, although the rate of free radical production was not controlled (5). Similar phenomenon was observed in photooxidation of PUFA in aqueous micelles (6). However, the reason for this reversed relationship in reactivity has not been adequately explained (5,6). In order to answer this question, we compare the oxidation kinetics of methyl eicosapentaenoate (20:5n-3) and methyl linoleate (18:2n-6) both in homogeneous chlorobenzene solution and in aqueous micelles using both water- and lipid-soluble azo initiators to obtain a constant rate of free radical production. Consumption of substrates was determined by gas chromatography (GC). Substrates were extracted from aqueous micelles with hexane/methanol (5:1, vol/vol) and condensed under reduced pressure prior to the injection.

When 200 mM 20:5n-3 and 18:2n-6 were independently oxidized with 10 mM lipid-soluble initiator, 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) in chlorobenzene, the rate of consumption of 20:5n-3 was about two times faster than that of 18:2n-6 as shown in Figure 1. On the other hand, the rate of disappearance of 20:5n-3 was about five times slower than that of 18:2n-6 when 1.6 mM 20:5n-3 and 18:2n-6 were independently oxidized with 2 mM AMVN in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM Triton X-100 (Fig. 2). This was also the case when the oxidation in aqueous micelles was initiated with water-soluble initiator, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). These data confirm the

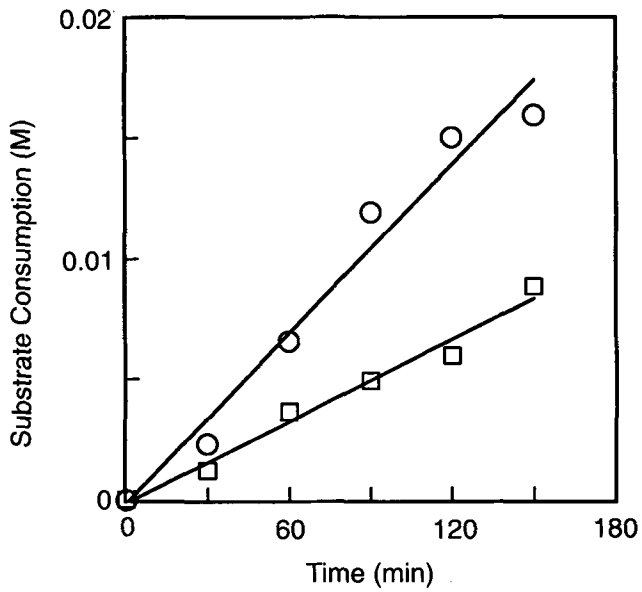


FIG. 1. Substrate consumption in 10 mM 2,2′-azobis(2,4-dimethylvaleronitrile)-initiated, aerobic oxidation of 200 mM 20:5n-3 (○) and 18:2n-6 (□) in chlorobenzene at 37°C. Oxidations were conducted separately.

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Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); 16:0, ethyl palmitate; GC, gas chromatography; 20:5n-3, methyl eicosapentaenoate; 18:2n-6, methyl linoleate; PUFA, polyunsaturated fatty acids.

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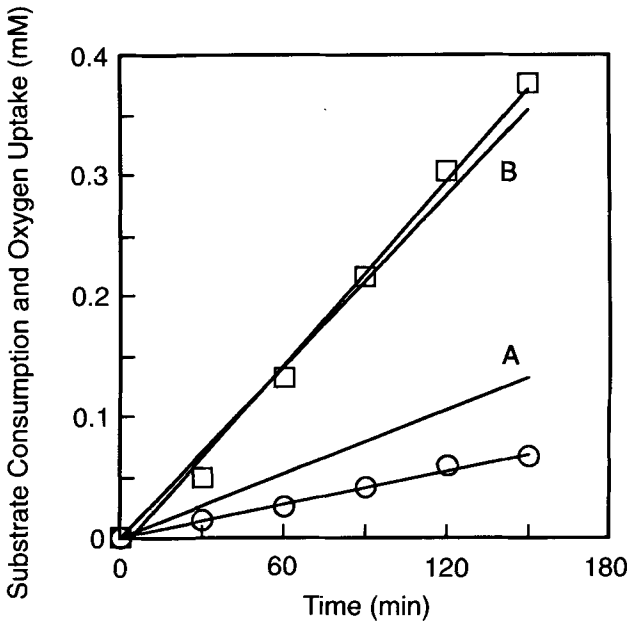


FIG. 2. Substrate consumption in 2.0 mM 2,2′-azobis(2,4-dimethylvaleronitrile)-initiated, aerobic oxidation of 1.6 mM 20:5n-3 (○) and 1.6 mM 18:2n-6 (□) in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM Triton X-100 at 37°C. Oxidations were conducted separately. Lines A and B show oxygen uptake by 20:5n-3 and 18:2n-6, respectively, during the oxidations in the same conditions.

reported observation (5) that in aqueous micelles the oxidizability of PUFA is inversely correlated with the number of bisallylic positions available for oxidation.

The oxidation of substrate (*LH*) initiated with an azo initiator (*A-N=N-A*) proceeds by the following mechanism (2–4):

initiation:

$$A\text{-}N{=}N\text{-}A \xrightarrow{k_d} 2eA\bullet + (1-e)A - A + N_2 \quad [1]$$

$$A\bullet + O_2 \rightarrow AOO\bullet \quad [2]$$

propagation:

$$AOO\bullet + LH \xrightarrow{O_2} AOOH + LOO\bullet \quad [3]$$

$$LOO\bullet + LH \xrightarrow{k_p} LOOH + L\bullet \quad [4]$$

$$L\bullet + O_2 \rightarrow LOO\bullet \quad [5]$$

termination:

$$2\,LOO\bullet \xrightarrow{k_t} \text{nonradical products} \quad [6]$$

where k_p and k_t are the rate constants for propagation and termination Reactions 4 and 6, respectively. The rate of chain initiation, R_i, is given in Equation 7 where k_d is the rate constant for the decomposition of azo initiator and e is the efficiency of free radical production. The rate of substrate consumption, $-d[LH]/dt$, is expressed by Equation 8.

$$R_i = 2e \cdot k_d[A\text{–}N{=}N\text{–}A] \quad [7]$$

$$-d[LH]/dt = kp(R_i/2k_t)^{1/2}[LH] + R_i \quad [8]$$

R_i in Equation 7 is determined by measuring the induction period (t_{inh}) due to the addition of the chainbreaking antioxidant, 2,6-di-*tert*-butyl-4-methylphenol and is calculated from Equation 9 (2–4,7). The $e \cdot k_d$ values at 37°C for AAPH and AMVN when 1.6 mM substrate was dissolved in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM Triton X-100 are 3.5×10^{-7} s^{-1} and 2.5×10^{-7} s^{-1}, respectively. These values were the same, irrespective of the lipid substrates, 20:5n-3 or 18:2n-6. We have used $e \cdot k_d$ value, 2.2×10^{-6} s^{-1}, for AMVN in chlorobenzene obtained previously (8).

$$R_i = 2[\text{2,6-di-}tert\text{-butyl-4-methylphenol}]/t_{inh} \quad [9]$$

The rate of substrate consumption was proportional to the substrate concentration and to the square root of R_i (data not shown), indicating that Equation 8 holds. The oxidizability values, $k_p/(2k_t)^{1/2}$, are given in Table 1 which summarizes the kinetic data. The oxidizability at each bisallylic site for 18:2n-6 is about two times larger than that for 20:5n-3 in chlorobenzene. On the other hand, in aqueous micelles, the oxidizability at bisallylic site for 18:2n-6 is about 20 times larger than that for 20:5n-3, indicating 20:5n-3 is less susceptible to oxidation than 18:2n-6. It is noteworthy that the oxidizability for each substrate was almost the same when the oxidation was initiated with either water- or lipid-soluble initiators. The oxidizability at bisallylic site for 18:2n-6 increased by a factor of 2 on going from chlorobenzene to micelles. Similar results were reported by the comparison of the oxidation kinetics of linoleic acid in chlorobenzene and in sodium dodecyl sulfate micelles (9). However, the oxidizability at each of bisallylic sites for 20:5n-3 decreased by a factor of 5 in comparison to chlorobenzene and micelles, indicating that the reactivity of peroxyl radical derived from 20:5n-3 changed substantially in aqueous micelles (see later discussion).

The oxidations of 20:5n-3 and 18:2n-6 were also followed

TABLE 1
Oxidizability, $k_p/(2k_t)^{1/2}$, for Methyl Linoleate (18:2n-6) and Methyl Eicosapentaenoate (20:5n-3) in Chlorobenzene and in 50 mM Tris-HCl Buffer (pH 7.4) Containing 10 mM Triton X-100 at 37°C Under Atmospheric Oxygen

			$10^3 \times k_p/(2k_t)^{1/2}$, $(M \cdot s)^{-1/2}$	
Substrate	Medium	Initiator[a]	Per molecule	[b]
18:2n-6	Solution[c]	AMVN	20	10
	Micelle[d]	AMVN	40	20
	Micelle[d]	AAPH	35	17
20:5n-3	Solution[c]	AMVN	43	5.4
	Micelle[d]	AMVN	7.5	0.9
	Micelle[d]	AAPH	8.5	1.1

[a]AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride.
[b]Per number of bisallylic hydrogens in a molecule.
[c]In chlorobenzene.
[d]Concentrations are those in oil region, and they are calculated assuming that the densities of 20:5n-3, 18:2n-6, and Triton X-100 are all 0.9.

by measuring oxygen uptake using an oxygen electrode in aqueous micelles. Figure 2 shows that the rates of oxygen uptake and substrate consumption were the same in the AMVN-initiated oxidation of 18:2n-6 in micelles. On the other hand, the ratio of the rate of oxygen uptake to that of the substrate consumption is about 2 in the oxidation of 20:5n-3. These data indicate that the oxidation of 20:5n-3 may proceed by a more complicated mechanism which includes the intramolecular cyclization of peroxyl radicals (10). This intramolecular reaction was shown to be evident in the oxidation of PUFA having more than three double bonds (10–12).

The peroxyl radical derived from 20:5n-3 should contain two molecules of absorbed oxygen. Therefore, this peroxyl radical is likely to be more polar than the peroxyl radical from 18:2n-6 having absorbed only one molecule of oxygen. Therefore, it is expected that the peroxyl radical derived from 20:5n-3 may diffuse from the hydrophobic region of the micelles to the polar surface region of the micelles as first suggested by Barclay and Ingold (13). This results in a decrease in the rate of propagation reaction since there may be more 20:5n-3 peroxyl radicals located at the surface as compared with the internal lipophilic region. Although Barclay *et al.* (9,14) reported a decrease in termination reactions for 18:2n-6 in micelles compared to organic solution, it is possible that 20:5n-3 peroxyl radicals may be more inclined toward termination reactions by their propensity to condense as polar groups at the surface layer of the micelles. Both changes would cause a decrease of oxidizability for 20:5n-3 in aqueous micelles as expected from Equation 8.

This interpretation was confirmed by the results of the oxidation of a 20:5n-3 and 18:2n-6 mixture (total 1.6 mM) in aqueous micelles. Table 2 shows that the relative reactivity of 20:5n-3 and 18:2n-6 toward peroxyl radical is about 3.5:1 (see (–*d*[20:5n-3]/*dt*/-*d*[18:2n-6]/*dt*)/([20:5n-3]/[18:2n-6]) values). This number is close to the ratio (4:1) of bisallylic sites for 20:5n-3 and 18:2n-6, suggesting 20:5n-3 is more prone to oxidation than 18:2n-6 even in aqueous micelles. However, the replacement of 18:2n-6 by 20:5n-3 (18:2n-6 to 20:5n-3 ratios of 1:7, 1:4, 1:1, and 0:1) reduced the rate of total substrate disappearance by factors of 3, 4, 5, and 5, respectively (Table 2). If the peroxyl radical derived from 20:5n-3 has similar reactivity in propagation and termination reactions as the peroxyl radical derived from 18:2n-6 in aqueous micelles as is observed in homogeneous solution, such a great reduction in the rate of total substrate disappearance should not be seen. Therefore, the results shown in Table 2 strongly suggest that the peroxyl radical derived from 20:5n-3 resides at a different location from where the peroxyl radical derived from 18:2n-6 is located in aqueous micelles. It is likely to be at the surface of the micelles since the peroxyl radical derived from 20:5n-3 is more polar than that from 18:2n-6. This would explain the reduction of oxidizability for 20:5n-3 in aqueous micelles by enhancing the rate of chain termination reaction of peroxyl radical and by reducing the rate of propagation since there may be more 20:5n-3 peroxyl radicals at the surface than in the micelle core.

TABLE 2
Aerobic Oxidation of Methyl Linoleate (18:2n-6) and Methyl Eicosapentaenoate (20:5n-3) in 50 mM Tris-HCl buffer (pH 7.4) Containing 10 mM Triton X-100 Initiated with 4 mM 2,2′-azobis(2-amidinopropane) Dihydrochloride (AAPH) at 37°C[a]

	Run number				
	1	2	3	4	5
[18:2n-6], mM	1.6	1.4	1.28	0.8	0.0
[20:5n-3], mM	0.0	0.2	0.32	0.8	1.6
[16:0][b], mM	4.0	4.0	4.0	4.0	4.0
-*d*[18:2n-6]/*dt*, nM/s	68.1	15.0	8.8	3.1	0.0
-*d*[20:5n-3]/*dt*, nM/s	0.0	7.1	8.0	11.2	14.1
-*d*[18:2n-6 + 20:5n-3]/*dt*, nM/s	68.1	22.1	16.8	14.3	14.1
[c]		3.31	3.64	3.61	

[a]Concentrations are expressed *in* aqueous micelles. R_i = 2.8 nM/s.
[b]Ethyl palmitate (16:0) was used as an internal standard.
[c]Ratio of oxidizabilities of 20:5-n-3 and 18:2n-6 (-*d*[20:5n-3]/*dt*/-*d*[18:2n-6]/*dt*)/([20:5n-3]/[18:2n-6]).

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Effects of Dietary Triacylglycerol Structure on Triacylglycerols of Resultant Chylomicrons from Fish Oil- and Seal Oil-Fed Rats

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ABSTRACT: We investigated the influence of the intramolecular fatty acid distribution of dietary triacyl-*sn*-glycerols (TAG) rich in n-3 polyunsaturated fatty acids (PUFA) on the structure of chylomicron TAG. Fish oil and seal oil, comparable in fatty acid compositions but with different contents of major n-3 PUFA esterified at the *sn*-2 position (20:5n-3, 46.6%, and 5.3%; 22:6n-3, 75.5%, and 3.8%, respectively), were fed to rats. Mesenteric lymph was collected and the chylomicrons were isolated by ultracentrifugation. The fatty acid composition of chylomicrons largely reflected the fatty acid composition of the oils administered. The intramolecular fatty acid distributions of the TAG fed were reflected in the chylomicron TAG as the fraction of the total contents observed in the *sn*-2 position of 20:5n-3 were 23.6 and 13.3%, and of 22:6n-3 were 30.6 and 5.4% for resultant chylomicrons following fish oil and seal oil administration, respectively. Thus, after seal oil administration, significant higher load of n-3 PUFA was esterified in the *sn*-1,3 positions of chylomicron TAG compared with fish oil administration ($P < 0.05$).

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Long-chain n-3 polyunsaturated fatty acids (PUFA), e.g., eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids, are of interest in general public health (1), as well as in enteral nutrition for hospital patients (2). *In vitro* studies have demonstrated that n-3 PUFA may exhibit resistance toward hydrolysis in the gastrointestinal tract (3,4), and it has thus been speculated that fish oil may not be efficiently digested and absorbed. The intestinal digestion of triacyl-*sn*-glycerol (TAG) is achieved mainly by the *sn*-1,3 specific pancreatic lipase. The 2-monoacyl-*sn*-glycerols (2-MAG) and free fatty acids formed are absorbed by the enterocytes and, once entering, are reacylated to TAG primarily by the 2-MAG pathway during fat absorption. Åkesson *et al.* (5) demonstrated that during absorption of 1,2-dilauryl-3-oleyl-*sn*-glycerol, lauric acid was maintained in the *sn*-2 position, and during absorption of menhaden oil, in which PUFA dominate in the *sn*-2 position, 85% of the fatty acids of the MAG obtained from the enterocytes were maintained in the TAG of chylomicrons (6). The intramolecular distributions of fatty acids in TAG from marine mammals and fish are different. In the seal oil, the n-3 PUFA are esterified in the *sn*-1,3 positions of the TAG, whereas in fish oils the PUFA are mostly esterified in the *sn*-2 position (4). This different distribution of fatty acids in the TAG may affect the intestinal absorption and distribution of the n-3 PUFA in humans (7). However, we have previously demonstrated that the absorption of n-3 PUFA from native oils, fish and seal oils, was affected by the intramolecular fatty acid distribution only to a minor degree in the rat (8). The intramolecular fatty acid distribution of TAG in chylomicrons may influence their metabolism in the systemic circulation, as the TAG are degraded by lipoprotein lipase which is an *sn*-1,3 specific lipase. Findings with chylomicrons produced during absorption of fish and seal oils demonstrated different metabolism, seal oil chylomicrons being removed faster from the plasma than fish oils chylomicrons (9). In the present paper, we investigated how the intramolecular distribution of n-3 PUFA in dietary TAG affects the intramolecular fatty acid distribution of TAG of chylomicrons.

MATERIALS AND METHODS

Male Wistar rats (specific-pathogen–free) were obtained from Møllegaard Laboratory (Ll. Skensved, Denmark). The rats were caged in pairs at 25°C at a relative humidity of 45%. Standard chow diet (standard chow diet/Altromin no. 1324; Chr. Pedersen, Ringsted, Denmark) and drinking water were provided *ad libitum*. The rats weighed 250–300 g at the time of surgery.

The surgical procedure and the experiment were approved by the Danish Committee on Animal Experiments. The rats were anesthetized with pentobarbital (7 mg/100 g body weight, i.p.), and a cannula was inserted into the main mesenteric lymph duct essentially as described by Bollmann *et al.* (10). Following surgery, the rats were placed in individual restraining cages in a quiet room (25°C) with free access to tap

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Abbreviations: MAG, monoacyl-*sn*-glycerols; PUFA, polyunsaturated fatty acids; TAG, triacyl-*sn*-glycerol.

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water. The rats were hydrated by infusion of physiological saline (0.15 M NaCl, 2 mL/h) through a gastric feeding tube placed at the time of surgery. The following day, 0.3 mL of either fish oil (Okholms fish oil, a product for human consumption; Thyborøn Andelsfiskeindustri, Thyborøn, Denmark) or seal oil (Greenlandic seal, *Pagophilus graenlandicus)*, followed by 0.5 mL of physiological saline (0.15 M NaCl), was administered through the feeding tube and the infusion of saline was continued. The lymph was discarded for the first 45 min, as no significant amount of dietary fat is transported through the lymphatics within that period, and then collected for eight hours. The chylomicrons were isolated by ultracentrifuge flotation using a discontinuous gradient (9,11), and the total lipid fraction extracted (12). The lipid extracts were applied to thin-layer chromatography plates (Merck art. no. 5721; Merck, Darmstadt, Germany) and developed in hexane/diethylether/acetic acid (80:20:1, by vol). The bands were visualized by spraying with 2,7-dichlorofluorescein, and the TAG band and application point containing the phospholipids were scraped off for fatty acid analysis. The fatty acids were methylated using KOH in methanol (13), and the fatty acid methyl esters analyzed by gas–liquid chromatography as previously described (9).

The intramolecular fatty acid distribution in TAG of dietary oils and chylomicrons was determined by Grignard degradation of the TAG using allylmagnesium bromide followed by analysis of the *sn*-2 MAG (14). This procedure eliminated the possible discrimination of n-3 PUFA due to slow hydrolysis previously reported for pancreatic lipase (3).

RESULTS

In the fish oil (Table 1), the major sources of n-3 PUFA were 20:5n-3 and 22:6n-3. The 22:6n-3 was concentrated in the *sn*-2 position of the TAG, whereas the 20:5n-3 was more evenly distributed between the *sn*-2 and the *sn*-1,3 positions of the TAG molecules. The 18:1, 20:1, and 22:1 were mainly esterified in the *sn*-1,3 positions, as reduced levels were found in the *sn*-2 position compared with the TAG, whereas approximately one-third of the saturated fatty acids were acylated in the *sn*-2 position. The intramolecular structure of seal oil TAG (Table 2) was significantly different from that of the fish oil TAG. The saturated and monounsaturated fatty acids with 14–18 carbons were mostly esterified in the *sn*-2 position, whereas 20:1 and 22:1 were esterified in the *sn*-1,3 positions. The PUFA, which mainly consisted of 20:5n-3 and 22:6n-3, were specifically esterified in the *sn*-1,3 positions, as only 5.3 and 3.9% of 20:5n-3 and 22:6n-3 of the total content in TAG, respectively, were found in the *sn*-2 position.

The fatty acid composition of the TAG and the phospholipids of chylomicrons and the intramolecular fatty acid distribution of the TAG following administration of oils were examined (Tables 1 and 2). The fatty acid composition of chylomicron TAG resembled the composition in the dietary oil. The ratio between mol% of n-3 PUFA in the chylomicron TAG and the oil administered was similar for the two oils

TABLE 1
The Fatty Acid Composition of Fish Oil, and Triacylglycerols and Phospholipids of the Resultant Chylomicrons

	Fish oil[a] (mol%)		Chylomicrons[b] (mol%)		
Fatty acid	TAG	*sn*-2 MAG[c]	TAG	*sn*-2 MAG[c]	PL
14:0	5.7	4.7	4.5 ± 0.2	3.4 ± 0.9	0.6 ± 0.1
16:0	17.3	18.8	19.8 ± 0.3	20.4 ± 1.6	23.7 ± 0.2
16:1	6.5	5.9	6.8 ± 0.1	5.6 ± 0.5	1.7 ± 0.1
18:0	2.8	1.0	3.6 ± 0.1	2.2 ± 0.4	17.6 ± 0.4
18:1	25.7	11.9	24.6 ± 1.0	26.9 ± 2.2	9.5 ± 0.5
18:2n-6	2.6	3.8	9.7 ± 0.4	15.7 ± 1.2	17.8 ± 0.5
18:3n-3	1.6	2.3	1.7 ± 0.1	1.9 ± 0.1	0.4 ± 0.1
18:4n-3	3.4	3.7	2.9 ± 0.4	2.3 ± 0.2	0.6 ± 0.1
20:1	3.7	1.4	2.6 ± 0.1	1.3 ± 0.2	0.7 ± 0.0
20:4n-6	0.7	0.9	3.0 ± 0.3	3.4 ± 0.6	16.7 ± 0.8
20:4n-3	0.7	0.7	0.2 ± 0.0	0.1 ± 0.1	0.1 ± 0.0
20:5n-3	9.8	13.7	6.5 ± 0.4	4.6 ± 0.3	4.4 ± 0.3
22:1	4.8	2.1	3.1 ± 0.2	1.3 ± 0.4	0.5 ± 0.0
22:5n-3	0.9	2.0	0.8 ± 0.0	0.8 ± 0.0	0.4 ± 0.0
22:6n-3	10.9	24.7	8.6 ± 0.5	7.9 ± 0.4	3.6 ± 0.2

[a]The data for the fish oil analyses represent average of three determinations for both the triacylglycerols (TAG) and *sn*-2-monoacylglycerols (*sn*-2 MAG).
[b]Chylomicrons were collected from six animals and the lipid fractions were extracted. Following separation on thin-layer chromatography, the TAG and phospholipid (PL) fractions were analyzed for fatty acid composition by gas–liquid chromatography. Data represent mean ± SEM.
[c]The fatty acid composition of the 2-MAG of the fish oil and the chylomicron TAG were determined by Grignard degradation followed by isolation and analysis of the *sn*-2 MAG. The fatty acid composition in the *sn*-1,3 position can be calculated using the equation: mol% in *sn*-1,3 = (3 × mol% in TAG –mol% in *sn*-2 MAG)/2.

TABLE 2
The Fatty Acid Composition of Seal Oil, and Triacylglycerols and Phospholipids of the Resultant Chylomicrons

	Seal oil[a] (mol%)		Chylomicrons[b] (mol%)		
Fatty acid	TAG	*sn*-2 MAG[c]	TAG	*sn*-2 MAG[c]	PL
14:0	4.9	7.4	3.4 ± 0.5	6.8 ± 0.9	0.6 ± 0.1
16:0	10.5	14.9	14.8 ± 0.6	19.9 ± 1.7	22.5 ± 2.5
16:1	18.7	30.3	16.1 ± 0.6	20.4 ± 0.9	3.7 ± 0.3
18:0	1.3	1.2	2.9 ± 0.1	2.2 ± 0.5	17.6 ± 0.5
18:1	24.3	31.0	21.8 ± 1.1	23.1 ± 2.0	9.5 ± 0.7
18:2n-6	1.3	2.6	8.8 ± 1.0	11.7 ± 2.1	18.4 ± 0.4
18:3n-3	0.5	0.9	0.8 ± 0.1	0.9 ± 0.1	0.2 ± 0.0
18:4n-3	1.7	0.9	1.6 ± 0.2	1.6 ± 0.8	0.7 ± 0.1
20:1	5.9	1.3	4.8 ± 0.3	2.5 ± 0.9	1.2 ± 0.1
20:4n-6	0.7	0.3	2.6 ± 0.3	1.7 ± 0.3	15.1 ± 0.5
20:4n-3	0.5	0.1	0.4 ± 0.1	0.4 ± 0.2	0.0 ± 0.0
20:5n-3	10.0	1.6	6.5 ± 0.4	2.6 ± 0.8	5.6 ± 0.4
22:1	2.0	0.2	1.2 ± 0.1	0.4 ± 0.3	0.2 ± 0.0
22:5n-3	4.0	0.4	3.1 ± 0.1	0.7 ± 0.3	0.5 ± 0.0
22:6n-3	9.5	1.1	8.0 ± 0.7	1.3 ± 0.2	2.4 ± 0.2

[a]The data for the seal oil analyses represent average of three determinations for both the TAG and *sn*-2 MAG. Abbreviations as in Table 1.
[b]Chylomicrons were collected from seven animals and the lipid fractions were extracted. Following separation on thin-layer chromatography, the TAG and PL fractions were analyzed for fatty acid composition by gas–liquid chromatography. Data represent mean ± SEM.
[c]The fatty acid composition of the 2-MAG of the fish oil and the chylomicron TAG were determined by Grignard degradation followed by isolation and analysis of the *sn*-2 MAG. The fatty acid composition in the *sn*-1,3 position can be calculated using the equation: mol% in *sn*-1,3 = (3 × mol% in TAG – mol% in *sn*-2 MAG)/2.

(~ 0.65 and ~0.80 for 20:5n-3 and 22:6n-3, respectively). The contents of 16:0, 18:2n-6, and 20:4n-6 in the chylomicron TAG were augmented compared with the content in the dietary oil, probably reflecting a contribution of fatty acids from the bile (15,16). The structural analysis of the chylomicron TAG showed that significantly more n-3 PUFA were esterified in the *sn*-1,3 positions after seal oil administration compared with fish oil administration ($P < 0.05$, Student's *t*-test).

Similarly, in the *sn*-2 position of the TAG from fish oil chylomicrons, a greater amount of n-3 PUFA was esterified compared with seal oil chylomicrons. The fatty acid composition of the chylomicron phospholipids reflected contribution of fatty acids of endogen origin as the contents of stearic acids (18:0, 17.6 ± 0.4, and 17.6 ± 0.5), linoleic acid (18:2n-6, 17.8 ± 0.5, and 18.4 ± 0.4), and arachidonic acid (20:4n-6, 16.7 ± 0.8, and 15.1 ± 0.5) in mol% following fish oil and seal oil administration, respectively, were high, whereas these fatty acids only were present in the oil in very low amounts. The fatty acids composition of the phospholipids from the two populations of chylomicrons was similar.

DISCUSSION

There has been no previous study comparing the effect of different intramolecular fatty acid distributions of n-3 PUFA in dietary TAG on the intramolecular fatty acid distribution of TAG of chylomicrons. Our data demonstrate that the ratio of n-3 PUFA esterified in the *sn*-2 vs. the *sn*-1,3 positions of chylomicron TAG depends on the oil administered. Following administration of seal oil, the n-3 PUFA were mainly esterified in the *sn*-1,3 positions of the chylomicron TAG, however, with an increase in the relative amount esterified in the *sn*-2 position from 5 mol% in the seal oil to 7.5 mol% the TAG of the chylomicrons. The n-3 PUFA from the fish oil were evenly distributed between the *sn*-2 and the *sn*-1,3 positions in chylomicron TAG. About one-third of the n-3 PUFA were esterified in the *sn*-2 position, compared with 57.5% in the oil administered. This agrees with the studies of Yang *et al.* (6,17), who observed a reduction in the percentage of the n-3 PUFA esterified in the *sn*-2 position of chylomicrons TAG compared to the menhaden oil administered. In contrast, Chernenko *et al.* (18) found that the positional distribution of fatty acids in the total lymph lipid fraction was similar to that in the fish oil (MaxEPA) administered. This discrepancy may reflect the use of total lymph and pancreatic lipase for structural analysis by Chernenko *et al.* (18). Alternatively, differences in the fatty acid composition of MaxEPA, 20:5n-3/22:6n-3 ~1.5, and the fish oil used in the present study, 20:5n-3/22:6n-3 ~0.9, may result in different metabolism of the n-3 PUFA.

A partial retailoring of the TAG during absorption reflects a contribution from the phosphatidic acid pathway in mucosa (19), the action of intestinal hydrolases (20), or a concerted action of lipases in the lumen (21), resulting in a total hydrolysis of some TAG to free fatty acids and glycerol. The similar fatty acids composition of chylomicron phospholipids following fish oil and seal oil administration and the similarities between the oils administered and the chylomicron TAG indicate that the phosphatidic pathway mainly contributed to phospholipid synthesized, whereas the TAG were synthesized by the MAG pathway. A reduction in long-chain fatty acids (20–22 carbon fatty acids) of the MAG from enterocytes compared with those recovered from the micellar phase of the lumen following digestion of menhaden oil (22) may reflect a total degradation of these MAG within the enterocytes or a special utilization of monoacylglycerols containing n-3 PUFA.

The predominant fatty acids in the phospholipids of chylomicrons were fatty acids of "endogenous?" origin, and the phospholipids seemed to transport the n-3 PUFA of dietary origin to a minor degree, especially when the amount of phospholipid (6–8 wt%) relative to TAG (90 wt%) in chylomicrons (23) was taken into consideration. The high levels of 20:5n-3 and 22:6n-3 found in the TAG of chylomicrons following fish oil and seal oil administration indicated that the n-3 PUFA were well absorbed under the present experimental conditions.

Our study demonstrates that n-3 PUFA are esterified at different positions within the TAG of chylomicrons following administration of different polyunsaturated oils, such as fish and seal oils. Extensive studies with model TAG, such as 1,2-dioleyl-3-stearyl-*sn*-glycerol and 1,3-dioleyl-2-stearyl-*sn*-glycerol, have demonstrated that the TAG structure affects the clearance of TAG and the removal of chylomicron remnants (24–26). Previously, we have demonstrated different metabolism of fish oil chylomicrons compared with seal oil chylomicrons. These findings may be explained by differences in the intramolecular fatty acid distribution of TAG due to either different hydrolysis rates of TAG by lipoprotein lipase or by different surface properties of the resultant 2-MAG affecting the metabolism of chylomicron particles.

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Dietary Long-Chain Polyunsaturated Fatty Acids Modify Heart, Kidney, and Lung Fatty Acid Composition in Weanling Rats

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ABSTRACT: The fatty acid composition of heart, kidney, and lung was studied in weanling rats fed three diets differing in their polyunsaturated fatty acid content for 0, 2, and 4 wk. The first group had a 10% w/w fat semipurified diet which consisted of a mixture of olive oil (62.5%), soybean oil (11.1%), and refined coconut oil (26.4%) and provided 18:1n-9, 18:2n-6, and 18:3n-3 in similar amounts to a maternal human milk (diet HO). The second group received 7% of HO fat and 3% fish oil (0.4% 20:4n-6 and 5% 22:6n-3 of total fatty acids) (diet FO), and the third group was fed 7% HO fat, 1.5% of the same fish oil, and 1.5% of a purified pig brain phospholipid concentrate (0.6% 20:4n-6 and 3.5% 22:6n-3 of total fatty acids) (diet FO + BPL). The experimental diets increased tissue monounsaturated fatty acids in comparison with rats at weaning. Tissue lipid content of 20:4n-6 was increased and 22:6n-3 decreased in Group HO compared with weanling rats, whereas opposite changes were observed in Group FO. Feeding diet FO + BPL increased 22:6n:3 in tissue lipids compared with diet HO, and increased 20:4n-6 content in relation to diet FO. Our results indicate that rat heart, kidney, and lung are highly responsive to dietary n-3 and n-6 long-chain polyunsaturated fatty acids during postnatal life.
Lipids 31, 345–348 (1996).

Deficient tissue 22:6n-3 accretion has been linked to abnormal electroretinograms in premature human infants (1), monkeys (2), and rats (3). Diets supplemented with n-3 long-chain polyunsaturated fatty acids (LC-PUFA) increase 22:6n-3 content in tissue lipids, and may affect visual function in infants (1) and learning ability in rats (4). However, prolonged feeding with n-3 LC-PUFA–supplemented formulas has been associated with lower head circumference in premature infants (5), reduced pup brain weight in rats (4), and decreased plasma and erythrocyte membrane 20:4n-6 in both neonates (6) and rats (7). In addition, a direct correlation between 20:4n-6 status of preterm infants and growth has been described (8). Feeding formulas supplemented with animal fat blends containing n-6 and n-3 LC-PUFA promoted acceptable levels of 20:4n-6 and 22:6n-3 in plasma and erythrocyte lipids in neonates (9) and in rats (10,11). However, the degree to which dietary 20:4n-6 and 22:6n-3 modify fatty acid composition of other tissues, such as heart, kidney, and lung, is unknown.

The aim of this study was to compare the fatty acid composition of heart, kidney, and lung in weanling rats fed on diets supplemented with preformed 20:4n-6 and 22:6n-3. Results on fatty acid composition of plasma lipids, erythrocyte membrane lipids, liver microsome phospholipids, and brain lipids have been previously described (10).

EXPERIMENTAL PROCEDURES

Animal experiments. Male Wistar rats at weaning (21 d) were purchased from Interfauna Iberica S.A. (Barcelona, Spain). Animals had no access to solid food. Seven rats were killed under anesthesia by terminal bleeding and used as control animals at weaning. The rest were randomly divided into three groups of 14 rats each. Rats received a 10% w/w fat semipurified diet and had free access to water. Group HO fat consisted of a mixture of olive oil (62.5%), soybean oil (11.1%), and refined coconut oil (26.4%) (devoid of LC-PUFA); group FO received 7% of group HO fat and 3% deodorized Spanish sardine oil (0.4% 20:4n-6 and 5.0% 22:6n-3 of total fatty acids); group FO + BPL was fed 7% group HO fat, 1.5% of the same fish oil concentrate, and 1.5% of a purified pig brain phospholipid concentrate rich in both 20:4n-6 and 22:6n-3 (0.6% 20:4n-6 and 3.5% 22:6n-3 of total fatty acids). The detailed composition of the diets has been previously described (10). Diets were prepared by the Research & Development Department of PULEVA S.A. (Granada, Spain).

After two and four weeks of feeding, seven animals from each group and feeding period were fasted for 24 h, anesthetized, and killed. Heart, kidney, and lung tissues were excised and processed immediately for extraction of total lipids (12). Fatty acids were methylated and separated by capillary gas chromatography as previously described (9).

Statistics. To evaluate the effect of three diets and two feeding periods, a two-way analysis of variance and a posteriori comparisons of means were done using the Bonferroni test (13). Comparisons with the baseline group were done

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Abbreviations: LC-PUFA, total long-chain polyunsaturated fatty acids equal or greater than 20 carbon atoms; MONO, total monounsaturated fatty acids; SAT, total saturated fatty acids.

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with the Dunnest test (13). The level of statistical significance was determined at $P < 0.05$.

RESULTS AND DISCUSSION

Tables 1 to 3 show the proportions of selected LC-PUFA and the sum of saturated fatty acids (SAT), monounsaturated fatty acids (MONO), and n-6 and n-3 LC-PUFA in heart, kidney, and lung lipids, respectively. No major differences were observed for SAT among groups and feeding periods except for group HO, which had significantly lower levels of SAT in heart at two and four weeks of feeding and in lung at two weeks in relation to weanling rats and group FO + BPL. Consistent with previous studies by our group of rats at weaning (10), the significant increases in MONO in heart, kidney, and lung lipids in all experimental groups compared with values at weaning are related to the relatively high content of 18:1n-9 in the diets, as it occurs in maternal human milk (14). Previous reports showed that high monounsaturated diets increased the levels of 18:1n-9 and MONO in erythrocyte membrane phospholipids in term infants (15) and in erythrocyte membrane phospholipids and liver microsomal phospholipids in rats at weaning (16).

After four weeks, group HO, which received a diet with an 18:2n-6/18:3n-3 ratio of 6:1 and devoid of LC-PUFA, had significantly lower total n-3 and total n-6 LC-PUFA values in heart, lung, and kidney lipids than rats at weaning. Within LC-PUFA, 20:4n-6 and 22:6n-3 were the major affected LC-PUFA in tissue lipids in group HO. These results suggest that the exclusive supply of 18:2n-6 and 18:3n-3 may not be sufficient to maintain tissue levels of 20:4n-6 and 22:6n-3 during postnatal life. The addition of fish oil to diet (group FO) significantly increased tissue 20:5n-3, 22:6n-3, and total n-3 LC-PUFA levels, whereas 20:4n-6 decreased in relation to diet HO and weanling rats.

The most interesting findings were that diet FO + BPL, which had a higher 20:4n-6 content than diet FO, increased not only heart, kidney, and lung 20:5n-3 and 22:6n-3, but also 20:4n-6 as compared with diet FO. Thus, a reduction of 22:6n-3/20:4n-6 ratio from 12.5 (diet FO) to 5.8 (diet FO + BPL) favored efficient 20:4n-6 incorporation to tissue lipids. Reduction of n-3 LC-PUFA content in diet FO + BPL could also result in a lower inhibition of 20:4n-6 synthesis from 18:2n-6 and incorporation into tissue lipids. Although our data do not allow us to make a conclusive statement on the beneficial or detrimental effects of dietary supplementation with n-3 and n-6 LC-PUFA on organ function during postnatal development, these results suggest that the level and type of dietary n-3 and n-6 fatty acids significantly modify tissue LC-PUFA in rats at weaning during postnatal life, and that incorporation of LC-PUFA to infant formulas should be carefully evaluated.

TABLE 1
Selected Fatty Acid Composition of Heart Lipids from Rats at Weaning Fed Diets Supplemented with Long-Chain Polyunsaturated Fatty Acids[a]

			Diets[b]		
Fatty acid	Weaning	Time	HO	FO	FO + BPL
20:4n-6	22.82 ± 0.54[d]	2	15.97 ± 1.21[e]	12.56 ± 0.37[f]	16.49 ± 0.55[c]
		4	21.29 ± 0.96[d]	12.03 ± 0.36[f]	15.68 ± 0.77[e]
20:5n-3	<0.20[h]	2	<0.20[h]	1.21 ± 0.06[e]	0.57 ± 0.04[g]
		4	<0.20[h]	1.43 ± 0.07[d]	0.71 ± 0.04[f]
22:6n-3	8.89 ± 0.52[f]	2	6.88 ± 0.49[g]	16.83 ± 0.66[d]	13.98 ± 0.56[e]
		4	8.13 ± 0.48[f,g]	18.32 ± 1.0[d]	14.60 ± 0.68[e]
SAT[c]	35.76 ± 0.24[d]	2	33.20 ± 0.32[e]	34.34 ± 0.36[d,e]	35.13 ± 0.28[d]
		4	33.89 ± 0.39[e]	34.29 ± 0.39[d,e]	35.43 ± 0.22[d]
MONO[c]	10.27 ± 1.06[f]	2	25.42 ± 2.81[d]	19.20 ± 0.93[e]	17.99 ± 0.86[e]
		4	23.32 ± 3.21[d,e]	18.50 ± 1.00[e]	20.73 ± 1.62[d,e]
n-6 LC-PUFA[c]	29.25 ± 0.96[d]	2	18.27 ± 1.45[f]	13.79 ± 0.39[g]	17.97 ± 0.57[f]
		4	23.88 ± 0.95[e]	12.98 ± 0.39[g]	17.07 ± 0.91[f]
n-3 LC-PUFA[c]	11.93 ± 0.65[f]	2	8.23 ± 0.60[g]	20.53 ± 0.75[d]	16.84 ± 0.70[e]
		4	9.44 ± 0.53[g]	22.00 ± 1.11[d]	17.66 ± 0.78[e]

[a]Results are expressed as mean percentages (mol/100 mol ± SEM) of total fatty acid methyl esters for seven animals in each group. Numbers with a different superscript (d–h) are significantly different, $P < 0.05$.
[b]Diet HO contained a 10% w/w fat mixture of olive oil (62.5%), soybean oil (11.1%), and refined coconut oil (26.4%); diet FO contained 7% of diet HO fat and 3% deodorized Spanish sardine oil; diet FO + BPL contained 7% of diet HO fat, 1.5% of the same fish oil concentrate, and 1.5% of a purified brain phospholipid concentrate.
[c]SAT, total saturated fatty acids; MONO, total monounsaturated fatty acids; LC-PUFA, total long-chain polyunsaturated fatty acids equal or greater than 20 carbon atoms.

TABLE 2
Selected Fatty Acid Composition of Kidney Lipids from Rats at Weaning Fed Diets Supplemented with Long-Chain Polyunsaturated Fatty Acids[a]

Fatty acid	Weaning	Time	Diets[b] HO	FO	FO + BPL
20:4n-6	27.44 ± 0.37[c]	2	22.28 ± 0.95[e]	15.97 ± 0.55[g]	19.43 ± 0.81[f]
		4	25.41 ± 0.50[d]	15.71 ± 0.66[g]	19.70 ± 0.50[f]
20:5n-3	<0.20[f]	2	0.26 ± 0.03[e]	3.41 ± 0.25[c]	2.03 ± 0.12[d]
		4	0.22 ± 0.02[e]	3.81 ± 0.36[c]	2.54 ± 0.15[d]
22:6n-3	2.83 ± 0.07[f]	2	1.64 ± 0.10[g]	4.91 ± 0.15[c]	3.40 ± 0.05[e]
		4	1.70 ± 0.05[g]	4.84 ± 0.27[c]	3.78 ± 0.11[d]
SAT[b]	40.14 ± 0.69[c]	2	38.81 ± 0.62[c]	39.54 ± 0.41[c]	39.95 ± 0.32[c]
		4	40.09 ± 0.23[c]	39.43 ± 0.56[c]	40.96 ± 0.33[c]
MONO[b]	15.37 ± 0.24[e]	2	24.69 ± 1.32[c]	23.64 ± 1.12[c,d]	21.62 ± 1.06[d]
		4	22.44 ± 0.65[c,d]	22.35 ± 1.13[c,d]	21.26 ± 0.42[d]
n-6 LC-PUFA[b]	31.04 ± 0.40[c]	2	24.58 ± 0.92[e]	17.40 ± 0.59[g]	21.02 ± 0.95[f]
		4	27.61 ± 0.53[d]	17.11 ± 0.57[g]	21.19 ± 0.52[f]
n-3 LC-PUFA[b]	3.72 ± 0.09[f]	2	2.23 ± 0.14[g]	9.60 ± 0.29[c]	6.26 ± 0.14[e]
		4	2.20 ± 0.08[g]	9.85 ± 0.52[c]	7.28 ± 0.22[d]

[a]Results are expressed as mean percentages (mol/100 mol ± SEM) of total fatty acid methyl esters for seven animals in each group. Numbers with a different superscript (c–g) are significantly different, $P < 0.05$.
[b]Diets and abbreviations as in Table 1.

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TABLE 3
Selected Fatty Acid Composition of Lung Lipids from Rats at Weaning Fed Diets Supplemented with Long-Chain Polyunsaturated Fatty Acids[a]

Fatty acid	Weaning	Time	Diets[b] HO	FO	FO + BPL
20:4n-6	13.15 ± 0.51[c]	2	8.43 ± 0.99[d,e]	4.90 ± 0.27[f]	6.63 ± 0.32[e]
		4	10.31 ± 0.53[d]	4.88 ± 0.30[f]	6.57 ± 0.37[e]
20:5n-3	<0.20[e]	2	<0.20[e]	2.37 ± 0.19[c]	1.19 ± 0.01[d]
		4	<0.20[e]	2.89 ± 0.17[c]	1.64 ± 0.11[d]
22:6n-3	1.59 ± 0.14[e]	2	1.62 ± 0.34[e]	4.08 ± 0.19[c]	3.16 ± 0.16[d]
		4	1.30 ± 0.09[e]	4.40 ± 0.25[c]	3.41 ± 0.17[d]
SAT[b]	45.97 ± 1.79[c,d]	2	41.79 ± 0.98[d]	43.50 ± 0.53[c,d]	44.19 ± 0.36[c]
		4	44.08 ± 0.60[c,d]	44.26 ± 0.71[c,d]	43.70 ± 0.60[c,d]
MONO[b]	23.15 ± 2.40[e]	2	39.57 ± 2.08[c]	35.90 ± 0.65[c,d]	35.54 ± 0.53[d]
		4	35.35 ± 1.23[c,d]	34.02 ± 1.27[d]	35.40 ± 1.39[c,d]
n-6 LC-PUFA[b]	18.49 ± 0.56[c]	2	11.42 ± 1.29[d]	6.28 ± 0.33[f]	8.53 ± 0.25[d]
		4	14.17 ± 0.75[d]	6.17 ± 0.34[f]	8.35 ± 0.46[d]
n-3 LC-PUFA[b]	2.97 ± 0.24[e]	2	2.80 ± 0.38[e,f]	9.25 ± 0.56[c]	6.41 ± 0.32[c]
		4	2.35 ± 0.11[f]	10.45 ± 0.58[c]	7.47 ± 0.38[c]

[a]Results are expressed as mean percentages (mol/100 mol ± SEM) of total fatty acid methyl esters for seven animals in each group. Numbers with a different superscript (c–f) are significantly different, $P < 0.05$.
[b]Diets and abbreviations as in Table 1.

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Reaction of Lipids Containing Hydroxy Groups with Trimethylsulfonium Hydroxide: Formation of *O*-Methyl Derivatives

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ABSTRACT: Base-catalyzed transesterification of acyl lipids with trimethylsulfonium hydroxide (TMSH) is an easy and convenient method for the preparation of fatty acid methyl esters (FAME) for gas chromatography (GC) analyses. We have found, however, that lipids containing hydroxy groups are partially converted to the corresponding *O*-methyl ether derivatives which may interfere with FAME in GC separations. For example, long-chain alcohols are found to be converted to alkyl methyl ethers, *rac*-1-*O*-alkylglycerols to the corresponding 2-*O*- and 3-*O*-monomethyl ethers, as well as 2,3-di-*O*-methyl ethers, hydroxy fatty acids to methoxy FAME, and cholesterol to cholesteryl 3β-methyl ether. From our results, it is obvious that TMSH derivatization method is not recommended without limitation for lipids containing hydroxy groups; it may be, however, of some diagnostic value for the analysis of such lipids by GC/mass spectrometry.

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Fatty acid methyl esters (FAME) are the most common derivatives for the analysis of fatty acids by gas chromatography (GC) (1,2). Recently, a very convenient method has been described for the derivatization of acyl lipids to FAME by using trimethylsulfonium hydroxide (TMSH) as the reagent (2–5). This method is based on earlier publications which have drawn attention to the advantage of this mild methylation reagent for the preparation of FAME for GC analysis (6,7). It was also shown that FAME are formed by base-catalyzed transesterification of acyl lipids such as triacylglycerols, whereas unesterified fatty acids are methylated by pyrolytic decomposition of the corresponding TMSH salts (7). Here we report about the limitations of the use of TMSH in derivatization of lipids for GC analyses in the case that appreciable proportions of lipids containing hydroxy groups are present in the sample.

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Abbreviations: FAME, fatty acid methyl esters; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; TMSH, trimethylsulfonium hydroxide.

MATERIALS AND METHODS

Chemicals. Long-chain alcohols, cholesterol, and methyl methanesulfonate were purchased from E. Merck (Darmstadt, Germany). Hydroxy and epoxy fatty acids, as well as cholesteryl 3β-methyl ether, were products of Sigma-Aldrich (Deisenhofen, Germany). TMSH reagent (0.2 M TMSH in methanol) was a product of Macherey-Nagel (Düren, Germany). Monomethylethers of *rac*-1-*O*-alkylglycerols were obtained from Calbiochem-Novabiochem (Bad Soden, Germany) and Berchtold (Bern, Switzerland).

Preparation of standards. FAME were prepared by the reaction of free fatty acids with diazomethane (8). *rac*-1-*O*-Alkylglycerols, *rac*-1-*O*-alkyl-2,3-di-*O*-methylglycerols, and methyl ethers of long-chain alcohols were prepared according to the procedure of Baumann and Mangold (9) by the use of alkyl methanesulfonates or methyl methanesulfonate. Methoxy FAME were prepared from the corresponding hydroxy FAME by the reaction with diazomethane in the presence of silica gel (10).

Derivatization for GC. To a solution of 0.8 mg lipid containing hydroxy groups in 40 μL *t*-butyl methyl ether, 20 μL TMSH reagent were added. The derivatization mixture was used immediately for GC.

GC. GC of was carried out using a Hewlett-Packard (Böblingen, Germany) HP-5890 Series II gas chromatograph fitted with a flame-ionization detector. *O*-Methyl ether derivatives of lipids were separated on a 0.15 μm FFAP-CB fused silica capillary column (Macherey-Nagel), 25 m × 0.25 mm i.d. The alkyl methyl ethers were separated using nitrogen as carrier gas (linear velocity 20 cm/s) initially at 160°C for 6 min, followed by linear programming from 160 to 240°C at 4°C per min. The final temperature was kept constant for 10 min. The split ratio was 1:10; the injector as well as flame-ionization detector temperature was 270°C. GC peaks were assigned by comparison of their retention times with those of known standards. Methoxy FAME as well as *O*-methyl derivatives of *rac*-1-*O*-alkylglycerols were separated under similar conditions using a linear temperature program from 160 to 240°C at 8°C per min. The final temperature was kept constant for 20 min. Cholesteryl 3β-methyl ether was separated isothermically at 240°C.

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GC/mass spectrometry (MS). GC/MS of *O*-methyl derivatives was performed at electron impact mode (70 eV) and chemical ionization mode (230 eV; methane as reagent gas, 160 Pa) on a Hewlett-Packard instrument Model 5890 Series II/5989 A (Palo Alto, CA). The GC instrument was equipped with a 0.23 μm Permabond OV-1 fused silica capillary column (Macherey-Nagel) 25 m × 0.32 mm i.d. The carrier gas was He at a flow rate of 1.0 mL/min. The column temperature was initially kept at 150°C for 5 min and then programmed from 150 to 270°C at 4°C/min; the final temperature was held for 5 min. Other operating conditions were split/splitless injector in splitless mode (temperature 300°C), interface temperature 280°C, and ion source temperature 200°C. Structural assignments were made for purchased genuine methoxy lipid standards and methoxy derivatives prepared from authentic samples by established procedures. Peaks of methoxy derivatives of hydroxy lipids obtained by the reaction with TMSH were assigned a structure if ions corresponding to all the important mass fragments of Table 1 were present, and no other high mass ions were detected.

RESULTS AND DISCUSSION

Single compounds or mixtures of lipids containing hydroxy groups, such as long-chain alcohols, alkylglycerols, hydroxy fatty acids, and cholesterol, were reacted with TMSH under pyrolytic conditions in order to study the possible formation of artefactual derivatives which may interfere with the GC analysis of FAME.

Figure 1 shows the gas chromatogram of the reaction products of a mixture of 1-hexadecanol and 1-octadecanol with TMSH. Obviously around 30% of the alcohols were transformed to the corresponding 1-*O*-methyl ethers, i.e., hexadecyl methyl ether and octadecyl methyl ether, which were

TABLE 1
Electron Impact Mass Spectral Data of *O*-Methyl Derivatives of Various Hydroxy Group Containing Lipids Formed by the Reaction with Trimethylsulfonium Hydroxide

O-Methyl ether derivatives of hydroxy group containing lipids	Important mass fragments (*m/z*)[a]			
Hexadecyl 1-*O*-methyl ether[b]	224 (14.3) $[M - CH_3OH]^+$	169 (5.0) $[M - (CH_3OH + C_2H_4)]^+$	168 (2.3) $[M - (CH_3OH + C_4H_8)]^+$	
Octadecyl 1-*O*-methyl ether[b]	252 (13.0) $[M - CH_3OH]^+$	224 (4.5) $[M - (CH_3OH + C_2H_4)]^+$	196 (1.8) $[M - (CH_3OH + C_4H_8)]^+$	
cis-9-Octadecenyl 1-*O*-methyl ether[b]	282 (1.3) $[M]^+$	250 (8.2) $[M - CH_3OH]^+$	222 (1.6) $[M - (CH_3OH + C_2H_4)]^+$	194 (2.2) $[M - (CH_3OH + C_4H_8)]^+$
rac-1-*O*-Hexadecyl-2,3-di-*O*-methylglycerol[c]	345 (0.3) $[M + 1]^+$	312 (1.9) $[M - CH_3OH]^+$	280 (1.9) $[M - C_2H_8O_2]^+$	253 (7.2) $[M - C_4H_{11}O_2]^+$
rac-1-*O*-octadecyl 2,3-di-*O*-methylglycerol[c]	373 (0.2) $[M + 1]^+$	340 (2.6) $[M - CH_3OH]^+$	308 (2.1) $[M - C_2H_8O_2]^+$	281 (9.2) $[M–C_4H_{11}O_2]^+$
rac-1-*O*-Octadec-9′-enyl-2,3-di-*O*-methylglycerol[c]	370 (1.1) $[M]^+$	293 (2.1) $[M - C_3H_9O_2]^+$	267 (1.5) $[M - C_5H_{11}O_2]^+$	250 (6.1) $[M - C_5H_{12}O_3]^+$
rac-1-*O*-Hexadecyl-2-*O*-methylglycerol[c]	331 (0.4) $[M + 1]^+$	285 (0.8) $[M - C_2H_5O]^+$	241 (1.3) $[M - C_4H_9O_2]^+$	222 (3.1)
rac-1-*O*-Octadecyl-2-*O*-methylglycerol[c]	359 (0.2) [M + 1]+	313 (1.5) $[M - C_2H_5O]^+$	269 (1.6) $[M - C_4H_9O_2]^+$	250 (4.6)
rac-1-*O*-Octadec-9′-enyl-2-*O*-methylglycerol[c]	356 (0.4) [M]$^+$	293 (1.4) $[M - C_2H_7O_2]^+$	267 (3.6) $[M - C_4H_9O_2]^+$	250 (11.3) $[M - C_4H_{10}O_3]^+$
rac-1-*O*-Hexadecyl-3-*O*-methylglycerol[c]	331 (1.0) $[M + 1]^+$	285 (1.7) $[M - C_2H_5O]^+$	253 (6.7) $[M - C_3H_9O_2]^+$	225 (6.3) $[M - C_4H_9O_3]^+$
rac-1-*O*-Octadecyl-3-*O*-methylglycerol[c]	359 (0.2) $[M + 1]^+$	313 (1.1) $[M - C_2H_5O]^+$	281 (5.8) $[M - C_3H_9O_2]^+$	253 (5.0) $[M - C_4H_9O_3]^+$
rac-1-*O*-Octadec-9′-enyl-3-*O*-methylglycerol[c]	356 (0.9) $[M + 1]^+$	293 (0.7) $[M - C_2H_7O_2]^+$	267 (1.4) $[M - C_4H_9O_2]^+$	250 (9.1) $[M - C_4H_{10}O_3]^+$
12-Methoxystearic acid methyl ester[d]	243 (65.3) $[M - C_6H_{13}]^+$	211 (15.8) $[243 - CH_3OH]^+$	174 (9.8) $[243 - (2 \times CH_3OH)]^+$	129 (66.3) $C_8H_{17}O^+$
12-Methoxyoctadec-9-enoic acid methyl ester[d]	294 (1.6) $[M - CH_3OH]^+$	129 (84.2) $C_8H_{17}O^+$	97 (84.7) $[129 - CH_3OH]^+$	
Cholesteryl 3β-*O*-methyl ether[e]	400 (50.5) $[M]^+$	368 (99.4) $[M - CH_3OH]^+$	353 (46.8) $[M - C_2H_7O]^+$	255 (32.9) $[M - (CH_3OH + C_8H_{17})]^+$

[a]Intensities relative to base peak.
[b]Base peaks of alkyl 1-*O*-methyl ethers: 16:0, *m/e* 55; 18:0, *m/e* 55; 18:1, *m/e* 57.
[c]Base peaks of isomeric mono- and dimethyl 1-*O*-alkylglyceryl ethers, respectively: 16:0, *m/e* 57 and 58; 18:0, *m/e* 57 and 58; 18:1, *m/e* 55 and 57.
[d]Base peaks of methoxy fatty acid methyl esters: 18:0, *m/e* 55; 18:1, *m/e* 55.
[e]Base peak of cholesteryl 3β-*O*-methyl ether: *m/e* 55.

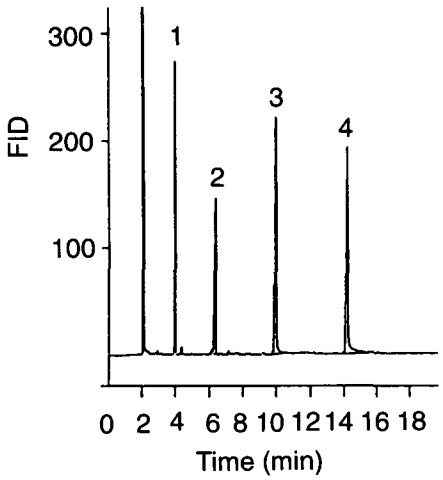


FIG. 1. Gas chromatogram of the reaction products of a mixture of 1-hexadecanol and 1-octadecanol with trimethylsulfonium hydroxide (1, hexadecyl 1-*O*-methyl ether; 2, octadecyl 1-*O*-methyl ether; 3, 1-hexadecanol, not reacted; 4, 1-octadecanol, not reacted); FID, flame-ionization detection.

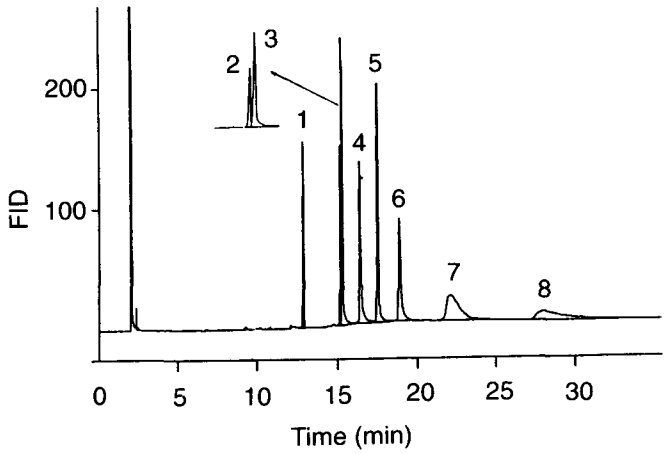


FIG. 2. Gas chromatogram of the reaction products of a mixture of *rac*-1-*O*-hexadecylglycerol and *rac*-1-*O*-octadecylglycerol with trimethylsulfonium hydroxide (1, *rac*-1-*O*-hexadecyl-2,3-di-*O*-methylglycerol; 2, *rac*-1-*O*-octadecyl-2,3-di-*O*-methylglycerol; 3, *rac*-1-*O*-hexadecyl-3-*O*-methylglycerol; 4, *rac*-1-*O*-hexadecyl-2-*O*-methylglycerol; 5, *rac*-1-*O*-octadecyl-3-*O*-methylglycerol; 6, *rac*-1-*O*-octadecyl-2-*O*-methylglycerol; 7, *rac*-1-*O*-hexadecylglycerol, not reacted; 8, *rac*-1-*O*-octadecylglycerol, not reacted); see Figure 1 for abbreviation.

identified by comparison with standards as well as by GC/MS (Table 1). The most important mass fragments for the structural assignment of the primary long-chain alcohols were the $[M\text{-}CH_3OH]^+$ ions which are typical of the *O*-methyl ether structure. Other structurally important intense ions have not been found. These observations are consistent with the literature (11). Molecular weight was determined in all samples, i.e., alkylmethyl ethers, alkylmethylglyceryl ethers, methoxy FAME, and cholesteryl 3β-*O*-methyl ether, by chemical ionization MS.

The gas chromatogram of the reaction products formed from a mixture of *rac*-1-*O*-hexadecylglycerol and *rac*-1-*O*-octadecylglycerol with TMSH is shown in Figure 2. The six reaction products observed in addition to the nonreacted alkylglycerols were identified by comparison with authentic standards as well as by GC/MS (Table 1) as the isomeric 2-*O*-methyl and 3-*O*-methyl 1-*O*-alkylglycerols, respectively, and the corresponding 2,3-di-*O*-methyl ether derivatives. In most electron impact spectra, small proportions of $[M + 1]^+$ ions have been observed. Earlier studies have also shown $[M + 1]^+$ ions for saturated long-chain alcohols (11), whereas $[M + 1]^+$ peaks have not been detected for alkylglyceryl ethers (12). Fragments with two, three, or four carbon atoms were found to be typical cleavage products of the glycerol backbone of alkyl(methyl)glyceryl ethers (Table 1) which is consistent with the literature (13). It is interesting to note that the 3-*O*-methyl derivatives are formed in higher proportions than the 2-*O*-methyl derivatives (ratio around 1:0.8; yield of mono- plus dimethyl derivatives around 80%), suggesting that different sterical effects are operative in the *O*-methylation at positions 2 and 3 of 1-*O*-alkylglycerols.

Figure 3 shows the gas chromatogram of the reaction products of a mixture of 12-hydroxystearic acid and ricinoleic acid with TMSH. Obviously, the free fatty acids were converted completely to the methyl esters. In addition, small proportions of methyl 12-methoxystearate and methyl 12-methoxyoleate (around 3 to 5%, each) are formed as confirmed by comparison with standards as well as by GC/MS (Table 1). The loss of CH_3OH fragments is typical of lipids containing methyl ether and methyl ester groups (14); the fragments at *m/e* 243, 211, 197, and 97 clearly show the position of the methoxy group at the acyl backbone. Surprisingly, both methyl *erythro*- and *threo*-9,10-dihydroxyoctadecanoates were con-

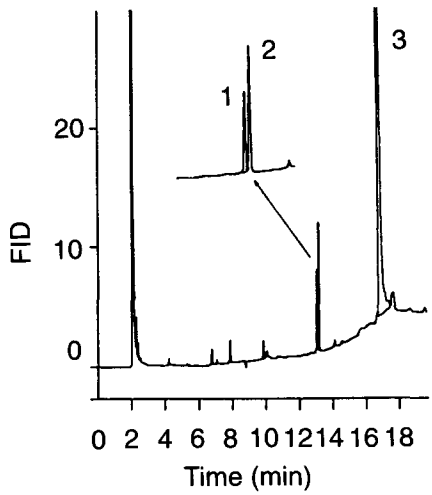


FIG. 3. Gas chromatogram of the reaction products of a mixture of 12-hydroxy stearic acid and ricinoleic acid with trimethylsulfonium hydroxide (1, 12-methoxy stearic acid methyl ester; 2, 12-methoxy octadec-9-enoic acid methyl ester; 3, 12-hydroxy stearic acid methyl ester plus ricinoleic acid methyl ester); see Figure 1 for abbreviation.

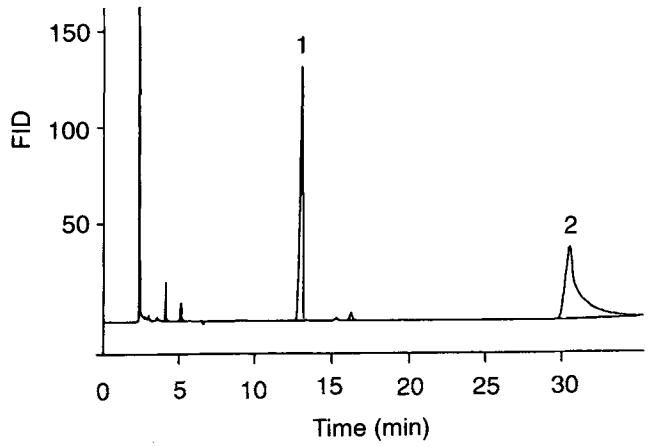


FIG. 4. Gas chromatogram of the reaction products of cholesterol with trimethylsulfonium hydroxide (1, cholesteryl 3β-*O*-methyl ether; 2, cholesterol, not reacted). See Figure 1 for abbreviation.

verted to the monomethoxy plus dimethoxy derivatives to an extent of around 60% (data not shown), demonstrating a positive effect of the vicinal hydroxy groups on *O*-methyl ether formation. A similar effect was observed for the vicinal hydroxy groups of 1-*O*-alkylglycerols.

It is evident from Figure 4 that cholesteryl 3β-*O*-methyl ether is formed in high proportions (around 40%) by reacting cholesterol with TMSH. The 3-*O*-methyl derivative was identified by comparison with an authentic standard as well as by GC/MS (Table 1). In addition, mass spectrum of cholesteryl 3β-*O*-methyl ether agreed with that given in the literature (15).

In conclusion, our data clearly show that *O*-methyl ether derivatives are formed, in part, from lipids containing hydroxy groups during pyrolytic reaction conditions in the GC injector, whereas the hydroxy groups do not react with the TMSH reagent at room temperature (data not shown). In GC, peaks of the *O*-methyl derivatives may interfere partially with peaks of FAME and, therefore, lead to erroneous results. The use of TMSH is not generally recommended, therefore, for the analysis of lipids containing hydroxy groups. It can be of some diagnostic value, however, for the detection of these lipids in samples. In contrast to lipids with hydroxy groups, epoxy fatty acids such as *cis*- and *trans*-9,10-epoxystearic acids are converted to the corresponding FAME without further derivatization (data not shown).

Studies are in progress concerning methods which enable us to circumvent the disturbing effects of *O*-methyl ether formation on FAME analyses of, e.g., wax esters, hydroxy fatty acids, and animal lipids with high proportions of ether glycerolipids.

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Effect of α-Tocopherol and Trolox on the Decomposition of Methyl Linoleate Hydroperoxides

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ABSTRACT: To clarify the mechanisms of antioxidant action, the effect of α-tocopherol and its water-soluble carboxylic acid derivative, Trolox, was studied on the decomposition of methyl linoleate hydroperoxides (MeLoOOH). Decomposition rate and the distribution of autoxidation products formed from MeLoOOH were followed by analyzing the volatile and nonvolatile products by static headspace gas chromatography and normal-phase high-performance liquid chromatography, respectively. Both α-tocopherol and Trolox markedly inhibited the decomposition of MeLoOOH in a concentration-dependent way. In the absence of antioxidants, MeLoOOH was completely decomposed after incubation for 48 h at 60°C, and in the presence of equal molar concentration of antioxidants only 6–7% of initial MeLoOOH was decomposed even after 280 h of incubation. MeLoOOH produced 1.2% methyl linoleate hydroxy compounds (MeLoOH) in the presence of α-tocopherol and 3.8% in the presence of Trolox. Both antioxidants inhibited the formation of volatile decomposition products and the formation of ketodiene compounds. The hydroxy compounds may be formed by the reaction of alkoxy radical and hydrogen donating antioxidants. Conversion of MeLoOOH into stable MeLoOH demonstrated that the antioxidants α-tocopherol and Trolox trap alkoxyl radicals by H-donation.

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Major focus of the research on antioxidants has been directed on their ability to inhibit hydroperoxide formation (1–3), but less attention has been given on their effectiveness to inhibit the decomposition of lipid hydroperoxides. Phenolic antioxidants, such as tocopherols, not only inhibit the formation of lipid hydroperoxides, but also are known to have an effect on the decomposition of hydroperoxides. At high concentration, α-tocopherol can suppress further reactions of methyl linoleate (MeLo) and linolenate monohydroperoxides by donating hydrogen to peroxyl radical, thus inhibiting formation of *trans,trans* monohydroperoxide isomers in MeLo and cyclic hydroperoxides in methyl linolenate (4,5). α-Tocopherol (6) and other phenolic antioxidants (7) modify the proportions of volatile decomposition products of methyl linoleate hydroperoxides (MeLoOOH) and reduce the total amount of volatile decomposition products. During autoxidation of corn oil, α-tocopherol reduced the formation of hexanal derived from linoleate hydroperoxides (8). Previous research on the effect of tocopherols on the decomposition of hydroperoxides is conflicting, and this may be due to different experimental conditions and concentration of antioxidants. In 2.5 mM aqueous solution of free linoleic acid, the addition of 0.625 μM–0.625 mM of α-, γ-, and δ-tocopherols increased the stability of linoleic acid hydroperoxides (9). However, addition of 7 μmoles α-tocopherol to MeLoOOH in benzene solution (75 μmoles in 30 mL representing 2.5 mM solution) did not influence the amount decomposed by Cu(II) ions (10). Previous work on hydroperoxide decomposition was performed mainly using metal or radical catalysts (10,11), or on fatty acids (9,12–15). Very little work has been reported on the effects of antioxidants on thermal decomposition of hydroperoxides. Because the secondary decomposition products are mainly responsible for causing rancidity in foods, as well as damage in biological material, it is also of high importance to study the effect of antioxidants on decomposition of hydroperoxides and the formation of secondary decomposition products.

The aim of this work was to clarify the mechanisms of α-tocopherol and its water-soluble derivative, Trolox, in inhibiting the formation of volatile and nonvolatile decomposition products from MeLoOOH. The formation of selected volatile and nonvolatile decomposition products was followed by static headspace gas chromatography (GC) and by normal-phase high-performance liquid chromatography (HPLC), respectively.

MATERIALS AND METHODS

Materials. MeLo was supplied by Nu-Chek-Prep, Inc. (Elysian, MN), hexadecane and α-tocopherol by Sigma Chemicals Co. (St. Louis, MO), and Trolox by Aldrich Chemical Company, Inc. (Milwaukee, WI). Hexane and diethyl ether were purchased commercially (Fisher Scientific, Fairlawn, NJ). All the solvents were HPLC grade.

Preparation of MeLoOOH. Hydroperoxides of MeLo were prepared from autoxidized MeLo by chromatography (16) (Sep-pak silica cartridge; Waters Chromatography Division, Milford, MA). The hydroperoxide fraction was shown to be

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Abbreviations: GC, gas chromatography; HPLC, high-performance liquid chromatography; MeLo, methyl linoleate; MeLoOH, methyl linoleate hydroxy compounds; MeLoOOH, methyl linoleate hydroperoxides.

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pure by thin-layer chromatography and had an absorptivity of 24,000 at 234 nm.

Decomposition of MeLoOOH. MeLoOOH or a 1:1 mixture of unoxidized MeLo and MeLoOOH (20 mM each) was dissolved in hexadecane solution in the absence or in the presence of 2 and 40 mM α-tocopherol or Trolox. MeLoOOH and α-tocopherol were soluble in hexadecane, but Trolox was only slightly soluble, and formed cloudy suspensions. Lipid solutions were incubated at 60°C for up to 282 h with continuous shaking. Sample aliquots for HPLC and GC analyses were taken at regular intervals.

HPLC analysis. Nonvolatile autoxidation products were analyzed by normal-phase HPLC, by a modified method of Tokita and Morita (17), using a Hewlett-Packard (Palo Alto, CA) model HP 1090 system equipped with a diode array detector. Particle size of the silica column was 5 μm (Supelco 25 cm × 2.1 mm; Bellefonte, PA). The solvent system was hexane/diethyl ether (85:15, vol/vol) with a flow rate of 0.3 mL/min. Ethanol used to stabilize diethyl ether was removed by silica column chromatography prior to the HPLC analysis. Autoxidation products were monitored at 233 nm (conjugated dienes), 210 nm (ester bond), and 268 nm (ketodienes). Tocopherols were monitored at 295 nm.

GC analysis of volatile decomposition products. Hexadecane solution (100 mg of 40 mM lipid solution) was weighed into 6-mL headspace vials. The hexanal formed was analyzed by static headspace capillary GC (16). The results were calculated as millimoles hexanal per kilogram hexadecane solution.

RESULTS

HPLC analysis of MeLoOOH decomposition products in the presence of α-tocopherol and Trolox. The main advantage of the HPLC method was the possibility of simultaneously monitoring MeLoOOH and formation of two important groups of decomposition products. The decomposition rate of hydroperoxides and formation of hydroxy compounds were monitored as total areas of four peaks (Fig. 1). Formation of ketodiene compounds was monitored as total areas of the two peaks in the chromatogram (Fig. 1A, peaks 1 and 2) having an absorption maximum at 268 nm in this solvent system (Fig. 2, spectrum 3), which is characteristic for ketodiene compounds (18). Total peak areas of these compounds were converted to percentage of the initial content of hydroperoxides by using the extinction coefficient of 28,600 for hydroperoxides (19), 27,200 for hydroxy compounds (19), and 28,000 for ketodiene compounds (18).

Elution order of the identified compounds was as follows: peaks 1 and 2, ketodienes; peaks 3–6, 13-*cis,trans* MeLoOOH, 13-*trans,trans* MeLoOOH, 9-*cis,trans* MeLoOOH, 9-*trans,trans* MeLoOOH; peaks 7–10, 13-*cis,trans* MeLoOH (methyl linoleate hydroxy compounds), 13-*trans,trans* MeLoOH, 9-*cis,trans* MeLoOH, 9-*trans,trans* MeLoOH (Fig. 1). Tentative identification of the MeLoOOH isomers was based on the ultraviolet spectra of the compounds (Fig. 2, spectras 1 and 2) and on the elution order reported in the literature (17,19). Retention times of the hydroxy compounds were confirmed with a reference sample prepared by reducing MeLoOOH mixture with $NaBH_4$. α-Tocopherol eluted before ketodienes (Fig. 1D, peak 11). Trolox could also be monitored by HPLC (Fig. 1C, peak 12). However, as it was only sparingly soluble in hexadecane, Trolox could not be measured quantitatively.

Effect of antioxidants on the decomposition of pure MeLoOOH. Both α-tocopherol and Trolox significantly inhibited the decomposition of MeLoOOH (Fig. 3). In the absence of antioxidants, 94% of initial MeLoOOH decomposed during the first 48 h at 60°C, whereas only 0–6% decomposed in the presence of antioxidants. After 282 h of incubation, MeLoOOH decomposed only 8.4% in the presence of 40 mM α-tocopherol and 11% in the presence of 40 mM Trolox. α-Tocopherol at 2 mM inhibited MeLoOOH decomposition up to 48 h, but Trolox was as effective at 2 mM as at 40 mM in inhibiting this decomposition for 282 h.

Antioxidants were detected in the samples until MeLoOOH started to decompose (Fig. 3). α-Tocopherol at 2 mM decomposed completely after 48 h incubation, and at 40 mM approximately 20% decomposed after 282 h incubation (Fig. 3B). Trolox was detected in the samples at 2 mM and 40 mM level, and samples also contained nonsolubilized Trolox after 282 h of incubation.

In the presence of antioxidants, the hydroxy derivatives (MeLoOH) produced represented 1.2–3.5% of initial MeLoOOH (Fig. 4A). Formation of MeLoOH was highest in the presence on 40 mM Trolox (3.5% of initial MeLoOOH), followed by 2 mM Trolox sample (2.1%) and 40 mM α-tocopherol (1.2%). With 40 mM Trolox, 1.5% MeLoOH was present initially after sample preparation. In the control sample, MeLoOH increased up to 0.5% during the first 20 h. However, the concentrations of MeLoOH compounds decreased rapidly in this sample during further incubation.

Ketodiene compounds were formed simultaneously with the rapid decomposition of hydroperoxides (Fig. 4B). In the control sample, ketodiene compounds increased from 0.3% of the initial MeLoOOH up to 4% after the first 20 h of incubation, and then decreased to 1.8% after 120 h of incubation. In the presence of α-tocopherol and Trolox, the formation of ketodiene compounds was inhibited together with the stabilization of hydroperoxides. Thus, after 282 h of incubation, 1.1% of ketodienes was formed in the presence of 40 mM Trolox, 0.9% in the 2 mM Trolox sample, and 1.6% in the 40 mM α-tocopherol sample (Table 1). In the presence of 2 mM α-tocopherol, the formation of ketodienes was inhibited during the first 48 h, increased rapidly to 2.8% when hydroperoxides started to decompose, and then decreased to 1.0% after 282 h of incubation.

Hexanal is a major volatile decomposition product of linoleate hydroperoxides (6,7). Both antioxidants inhibited hexanal formation effectively (Fig. 4C). Hexanal content increased initially in the control sample. In the presence of 2 mM α-tocopherol, hexanal increased simultaneously with

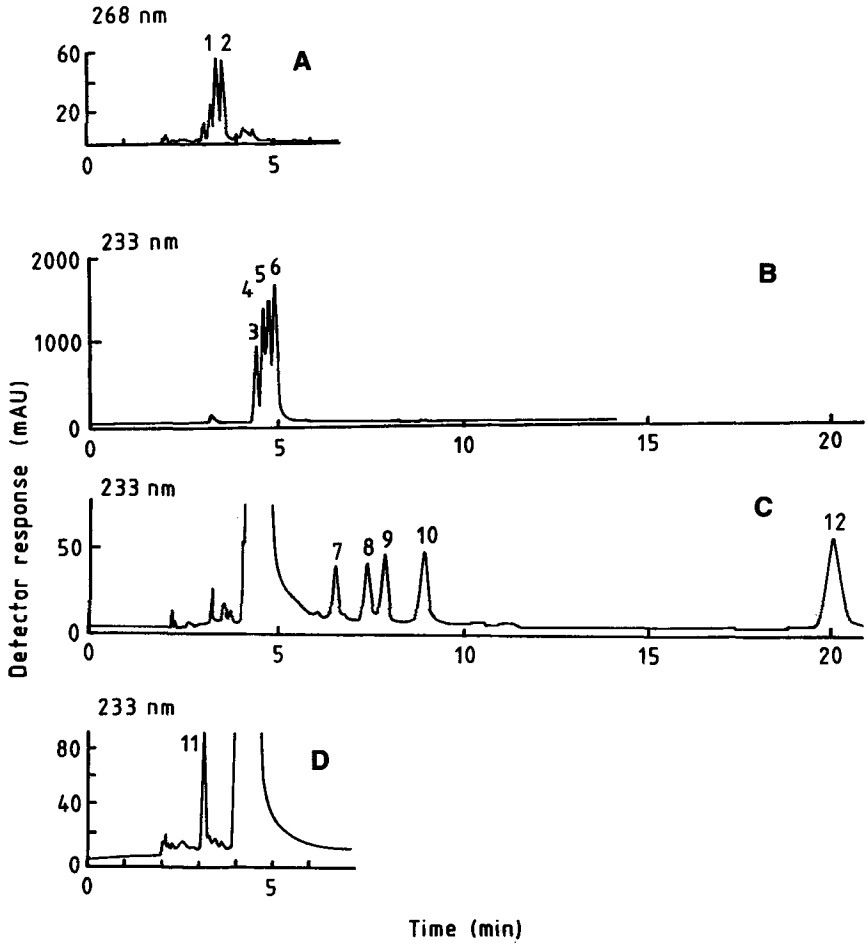


FIG. 1. High-performance liquid chromatographic (HPLC) chromatogram of oxidation products in the presence of antioxidants. Chromatographic conditions: silica column (5 μm, 25 cm × 2.1 mm), hexane/diethyl ether (85:15, vol/vol), flow rate of 0.3 mL/min. Autoxidation products were monitored at 233 nm (conjugated dienes) and 268 nm (ketodienes), and tocopherols and Trolox at 295 nm. Elution order of oxidation products: A, peaks 1 and 2, ketodienes; B, peaks 3–6, 13-*cis,trans* methyl linoleate hydroperoxides (MeLoOOH), 13-*trans,trans* MeLoOOH, 9-*cis,trans* MeLoOOH, 9-*trans,trans* MeLoOOH; C, peaks 7–10, 13-*cis,trans* methyl linoleate hydroxy compounds (MeLoOH), 13-*trans,trans* MeLoOH, 9-*cis,trans* MeLoOH, 9-*trans,trans* MeLoOH. Elution order of antioxidants: C, peak 12, Trolox; D, peak 11, α-tocopherol.

MeLoOOH decomposition after a lag period of 48 h. Hexanal formation was completely inhibited in the presence of 2 mM Trolox or 40 mM α-tocopherol and Trolox. After 282 h incubation, only 0.06 to 0.13 mmol/kg of hexanal was formed in the presence of 40 mM α-tocopherol or Trolox, compared to 2.24 mmol/kg after 48 h incubation in the absence of antioxidants.

Effect of antioxidants on the MeLo/MeLoOOH (1:1) mixture. The influence of unsaturated fatty acids as competitive hydrogen donors on the secondary reactions of hydroperoxides was investigated by decomposing MeLoOOH in the presence of unoxidized MeLo. In a mixture of MeLo (20 mM) and MeLoOOH (20 mM), changes in the rate of MeLoOOH decomposition, as well as formation of hexanal, MeLoOH, and ketodiene were similar, but lower than in pure MeLoOOH. After 282 h incubation, the amount of undecomposed MeLoOOH was 90.6% with 40 mM α-tocopherol, 88.9% with 40 mM Trolox, and 83.3% with 2 mM Trolox (Table 1). Oxidation of MeLo occurred in the sample containing 40 mM α-tocopherol, and the hydroperoxide content increased from 100% initially up to 123% during the first 114 h of incubation (Fig. 5A). α-Tocopherol apparently had a prooxidant effect at the 40 mM level. In the presence of 2 mM α-tocopherol, the hydroperoxides started to decompose after 114 h, and after 282 h of incubation only 15% of initial MeLoOOH was left undecomposed. Also, α-tocopherol was consumed after 120 h (Fig. 5B).

As with pure MeLoOOH, in the presence of MeLo, MeLoOH was produced in the presence of α-tocopherol and Trolox (Fig. 6A). After 330 h incubation, 3.17% MeLoOH

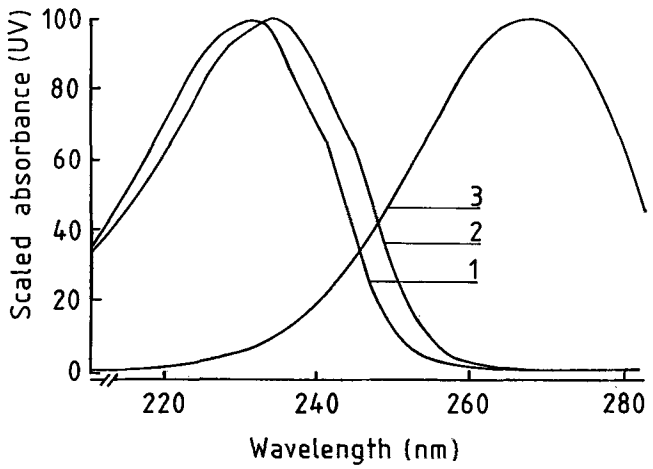


FIG. 2. Ultraviolet (UV) spectra of *trans,trans* (spectrum 1, combined peaks 4 and 6 in Fig. 1B) and *cis,trans* (spectrum 2, combined peaks 3 and 5 in Fig. 1B) hydroperoxides with absorption maximum of 230 and 235 nm, respectively, and ketodiene compounds (spectrum 3, combined peaks 1 and 2 in Fig. 1A) with absorption maximum at 268 nm.

was formed with 40 mM Trolox, 1.55% with 2 mM Trolox, and 0.54% with 40 mM α-tocopherol. In the presence of 2 mM α-tocopherol, MeLoOH content increased up to 0.54% after 282 h, but decreased after 330 h of incubation. Also in the MeLoOOH/MeLo mixture, the formation of ketodienes (Fig. 6B) and hexanal (Fig. 6C) increased together with MeLoOOH decomposition. Thus, ketodiene formation was highest in the control sample, reaching a maximum value of 5.6% after 48 h and 5.26% in the presence of 2 mM of α-tocopherol after 282 h of incubation. Ketodiene compounds were not formed initially in the presence of 40 mM α-tocopherol, 40 mM Trolox, or 2 mM Trolox. After 282 h incubation, 2.0% ketodiene compounds were formed in the presence of 40 mM α-tocopherol, 2.0% in 40 mM Trolox, and 1.5% in the presence of 2 mM Trolox (Fig. 6B).

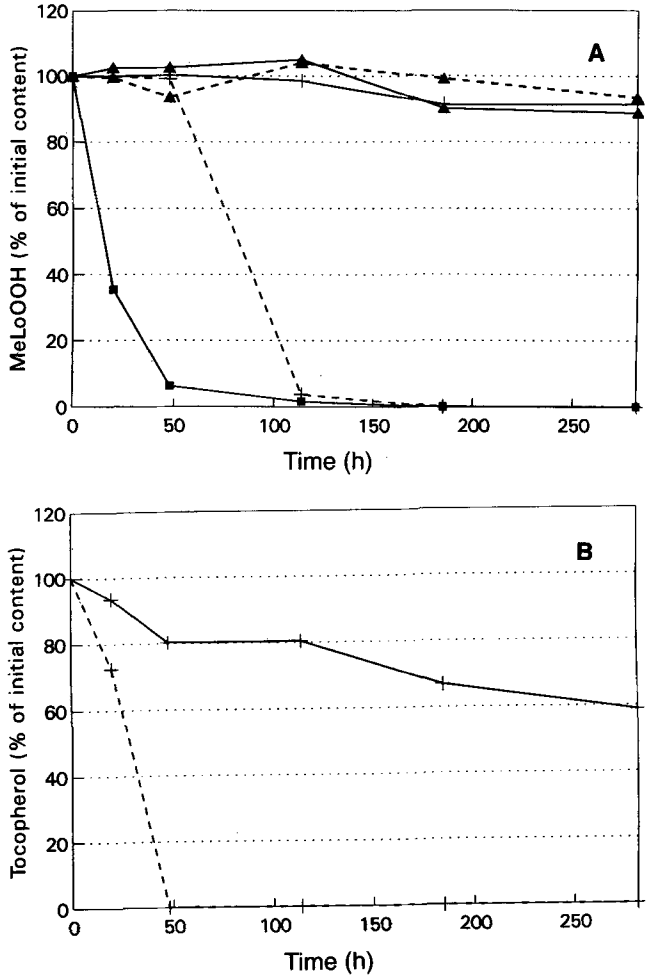


FIG. 3. Decomposition of methyl linoleate hydroperoxides (MeLoOOH) with or without antioxidant at 60°C in hexadecane solution: A, MeLoOOH; B, α-tocopherol. Legend: —■— = 40 mM MeLoOOH; —+— = 40 mM MeLoOOH + 40 mM α-tocopherol; ---+--- = 40 mM MeLoOOH + 2 mM α-tocopherol; —▲— = 40 mM MeLoOOH + 40 mM Trolox; ---▲--- = 40 mM MeLoOOH + 2 mM Trolox.

TABLE 1
Reaction Products Identified from the Decomposition of Methyl Linoleate Hydroperoxides After 282 h Incubation[a]

			Proportion of conversion products		
Sample	MeLOOH[b] (wt%)	Total conversion (wt%)	MeLoOH (%)	Ketodienes (%)	Conversion products[c] (%)
MeLoOOH (40 mM)	0	100	0	0.6	0.6
+ 40 mM α-Tocopherol	91.6	8.4	13.8	19.3	33.1
+ 2 mM α-Tocopherol	0	100	0	1.0	1.0
+ 40 mM Trolox	89.0	11.0	31.9	10.0	41.9
+ 2 mM Trolox	93.6	6.4	32.0	13.6	45.6
MeLoOOH/MeLo (20 mM each)	0	100	0	1.8	1.8
+ 40 mM α-Tocopherol	90.6	9.4	5.7	21.6	27.3
+ 2 mM α-Tocopherol	7.6	92.4	0	5.7	5.7
+ 40 mM Trolox	88.9	11.1	28.6	17.7	46.3
+ 2 mM Trolox	83.3	16.7	9.3	8.9	18.2

[a]MeLOOH, methyl linoleate hydroperoxides; MeLoOH, methyl linoleate hydroxy compounds; MeLo, methyl linleate. [b]Percentage of initial MeLOOH content. [c]All conversion products identified.

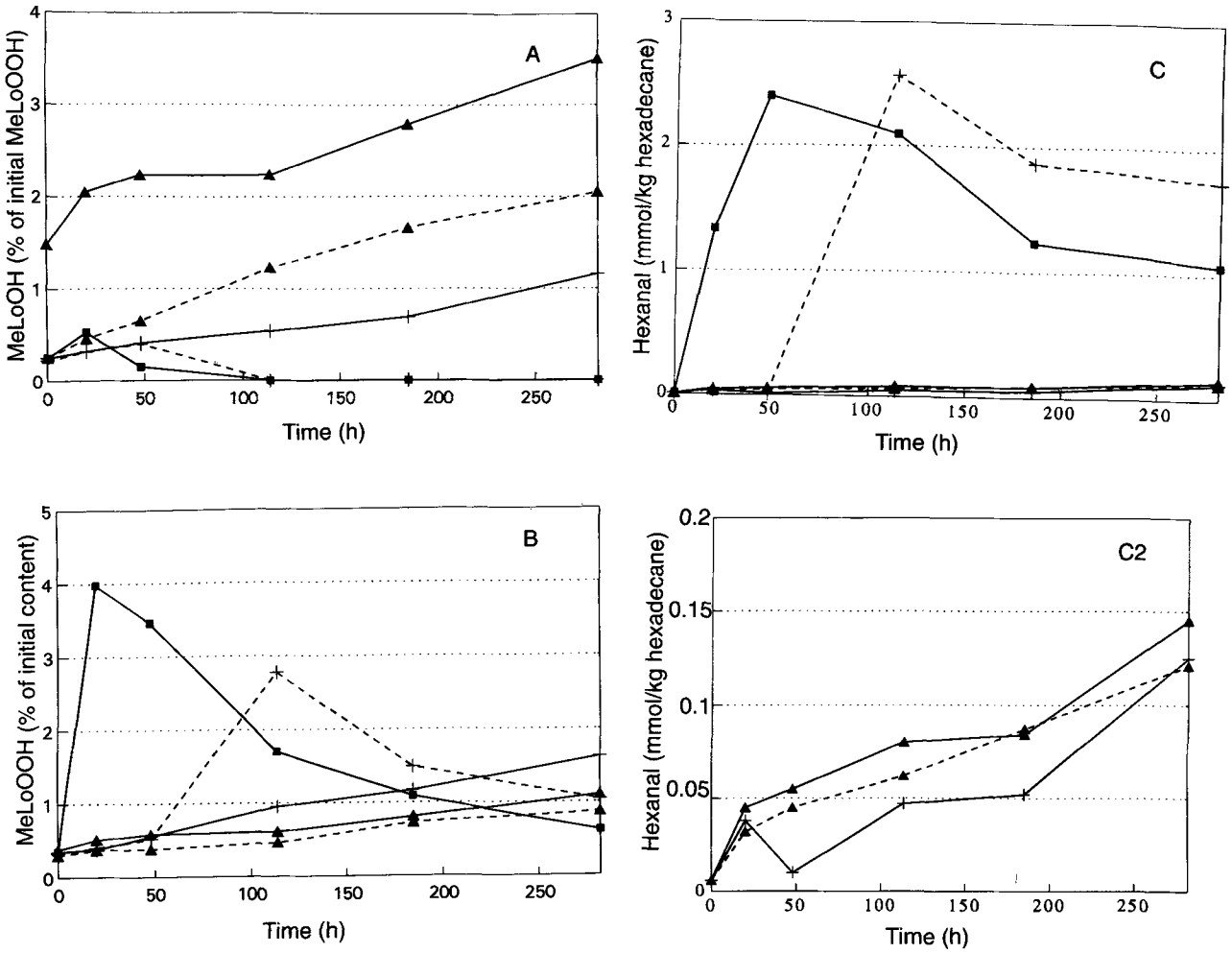


FIG. 4. A, Formation of hydroxy compounds; B, ketodiene compounds; C, hexanal, during decomposition of methyl linoleate hydroperoxides with or without antioxidant; C2, (C) with expanded y-axis. Samples in hexadecane solution. Legend as in Figure 3.

In the samples containing 40 mM α-tocopherol or Trolox and 2 mM Trolox, MeLoOH and ketodiene compounds were the major decomposition products of MeLoOOH, whereas in the highly decomposed samples a major part of the decomposition products was not detectable by the HPLC method used (Table 1). Thus, the conversion products that remained unidentified were 67% with 40 mM α-tocopherol, 99% with 2 mM α-tocopherol, 58% with 40 mM Trolox, and 54% with 2 mM Trolox (Table 1). More work is needed to further identify decomposition products other than hydroxy and ketodiene compounds.

DISCUSSION

The literature on decomposition of hydroperoxides deals mostly with lipids in the absence of antioxidants (10–15, 20,21). However, the pathways of decomposition would be expected to be markedly affected by the presence of hydrogen donating antioxidants. Decomposition of MeLoOOH leads to the same major products, such as ketodienes, hydroxy, epoxy and dimeric compounds, but in different ratios depending on whether the reaction proceeds through alkoxyl or peroxyl radical intermediates (10). Decomposition pathways of MeLoOOH depend markedly on the reaction conditions, and several mechanisms for lipid hydroperoxide decomposition have been suggested. According to the bond dissociation energies, the thermal decomposition of MeLoOOH may occur mainly through alkoxyl radical (44 kcal/mol) rather than through peroxyl radical (90 kcal/mol). However, the presence of transition metal ions may greatly affect the decomposition pathways. Peroxyl radicals may be produced by metal catalysts in their higher valence state, and alkoxyl radicals may be produced by metals in their lower valence state (22). MeLoOOH also have been shown to rearrange and decompose through pentadienyl radical (23). In this study no metal catalyst was added, but traces of metals are expected to be present in the reaction mixtures that would accelerate the decomposition of MeLoOOH at 60°C. Therefore, decomposition through alkoxyl radical is considered to be an important pathway.

The results of this study indicate that the phenolic antioxidants α-tocopherol and Trolox at high concentrations effi-

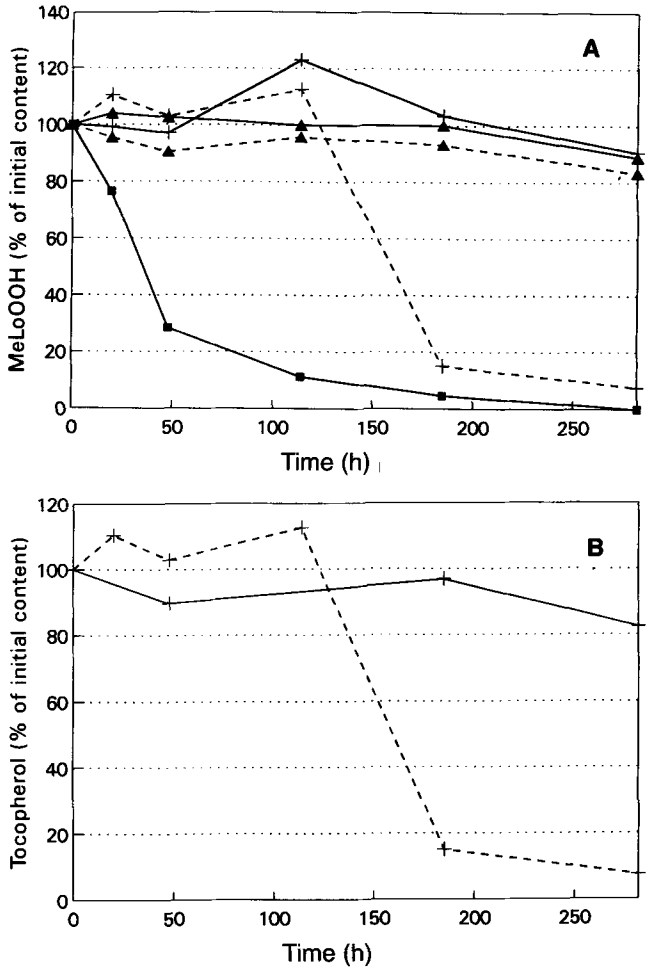


FIG. 5. Decomposition of methyl linoleate (MeLo) hydroperoxides (MeLoOOH) (A), and α-tocopherol (B), at 60°C. MeLoOOH/MeLo (20 mM each, 1:1) in hexadecane solution with or without antioxidants. Legend: —■— = 20 mM each of MeLoOOH and MeLo; —+— = 20 mM each of MeLoOOH and MeLo + 40 mM α-tocopherol; ---+--- = 20 mM each of MeLoOOH and MeLo + 2 mM α-tocopherol; —▲— = 20 mM each of MeLoOOH and MeLo + 40 mM Trolox; ---▲--- = 20 mM each of MeLoOOH and MeLo + 2 mM Trolox.

ciently inhibit the decomposition of MeLoOOH. This effect of α-tocopherol has been reported previously (6,9), but not for Trolox. This study provides, for the first time, evidence for the strong stabilizing effect of Trolox on lipid hydroperoxides. α-Tocopherol has been shown to inhibit decomposition of hydroperoxides in corn oil (8) and linoleic acid (9). Frankel and Gardner (6) observed that α-tocopherol at high concentrations decreases the total volatile products of MeLoOOH formed by thermal decomposition. They suggested that α-tocopherol at high concentration inhibits β-scission of alkoxyl radical (Eq. 1), leading to formation of aldehydes and hydrocarbons, by hydrogen donation.

$$R_2CH\text{–}O^{\bullet} \rightarrow RCHO + R^{\bullet} \quad [1]$$

Donation of the readily available hydrogen of α-tocopherol to alkoxyl radical would lead to formation of hydroxy compound (Eq. 2), through the following mechanism postulated by Bell *et al.* (24) and later by Hamberg (13) and Gardner (14):

$$RO^{\bullet} + RH \rightarrow ROH + R^{\bullet} \quad [2]$$

Because the oxy radical of an alkoxy is allylic and the oxy radical is located at a secondary carbon, straight abstraction of hydrogen (Eq. 2) is considered to be fairly insignificant in the absence of strong hydrogen donors (25). β-Scission (Eq. 1) producing aldehydes and intramolecular rearrangements producing epoxy compounds and dimers, as well as disproportionation, are considered more probable reaction pathways (25,26). However, strong hydrogen donors, such as α-tocopherol and Trolox, may markedly affect the reaction pathway involving alkoxy radical, and the conversion of MeLoOOH into MeLoOH would be favored, as observed in this study. Previously, Schieberle and Grosch (10) observed that the decomposition of MeLoOOH was inhibited by ascorbyl palmitate with the simultaneous formation of MeLoOH. Formation of MeLoOH was also detected during autoxidation of MeLo in the presence of ascorbyl palmitate (27).

In the present study, the decomposition of MeLoOOH was effectively inhibited by Trolox and α-tocopherol with the simultaneous formation of MeLoOH and no hexanal formation. These results support the hypothesis that in the presence of the strong hydrogen donors α-tocopherol and Trolox, hydrogen abstraction by alkoxyl radicals (Eq. 2) becomes more favorable than β-scission of alkoxyl radicals (Eq. 1), resulting in effective inhibition of hexanal formation. Because MeLoOH formation also increased in the presence of unsaturated fatty acid, which is a competitive hydrogen donor in lipid systems (28), the hydrogen source in the reaction is mainly the antioxidant (AH) (Eq. 3) rather than the unsaturated lipid (RH) (Eq. 2).

$$RO^{\bullet} + AH \rightarrow ROH + A^{\bullet} \quad [3]$$

Thus, the conversion of MeLoOOH into MeLoOH shown in this study may provide a pathway to explain the antioxidant mechanism of α-tocopherol and Trolox in inhibiting the decomposition of hydroperoxides.

Ketodiene compounds, 13- and 9-oxo octadecadienoic acids are one major group of products known from the homolytic decomposition of linoleic acid hydroperoxides (10–13,29). Ketodienes were the main breakdown products formed during the aerobic breakdown of MeLoOOH in the presence of copper through alkoxy radical intermediate (10). Proposed mechanisms for ketodiene formation include the disproportionation of peroxyl radicals through a tetroxide intermediate in which hydrogen atom is transferred from one radical to another (30) (Eq. 4), the elimination of hydrogen from a tetroxide intermediate in which only ketodiene compounds are formed (10) (Eq. 5), and the elimination of hydrogen from an alkoxyl radical (13,14) (Eq. 6).

$$2\,R_2CHOO^{\bullet} \rightarrow R_2\text{–}CH\text{–}OOOO\text{–}HCR_2 \rightarrow R_2CHOH + R_2C{=}O + O_2 \quad [4]$$

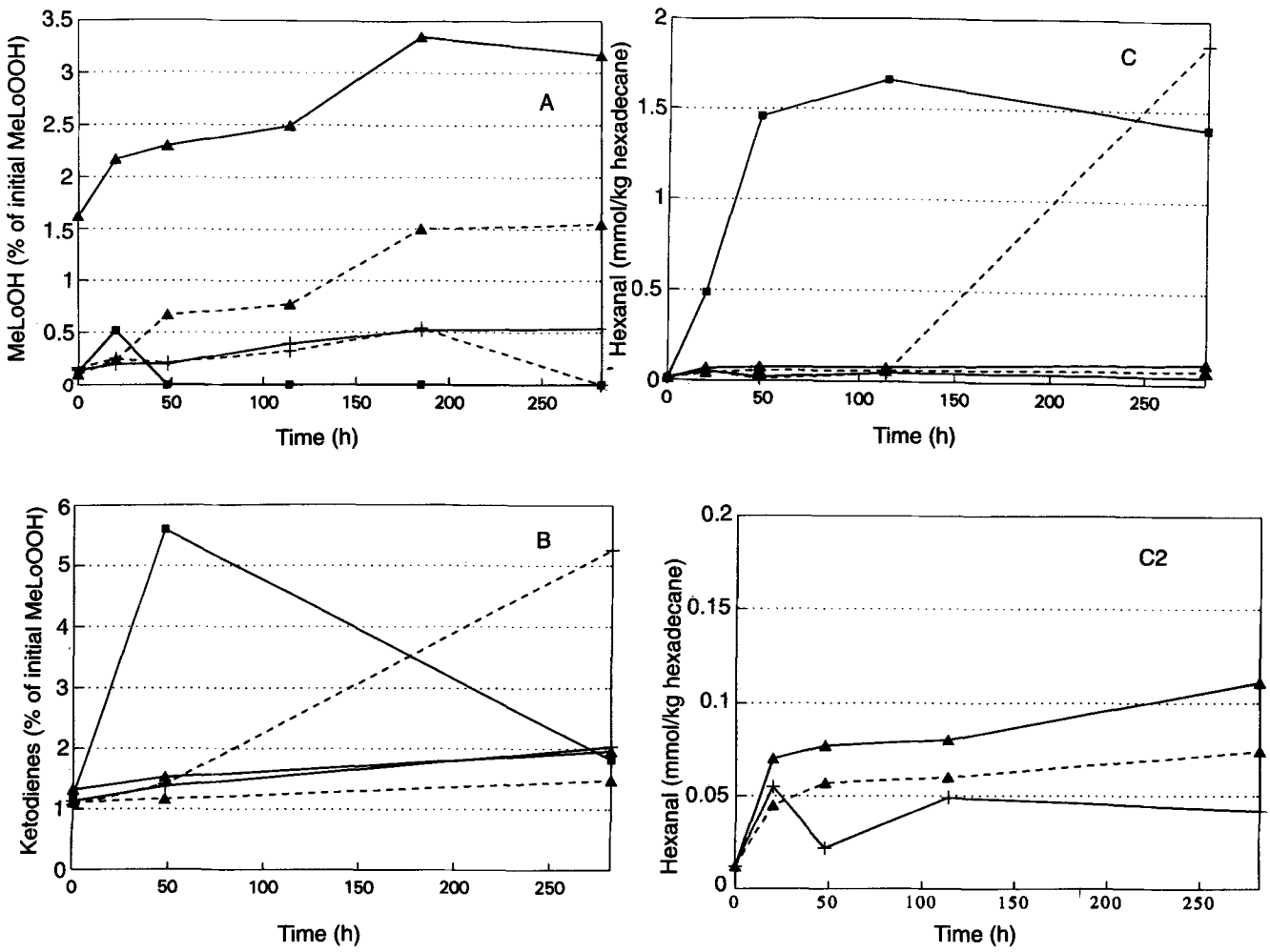


FIG. 6. A, Formation of hydroxy compounds; B, ketodiene compounds; C, hexanal, during decomposition of MeLoOOH with or without antioxidants; and C2, (C) with expanded y-axis. 40 mM solution of MeLoOOH/MeLo (1:1) in hexadecane solution. Legend and abbreviations as in Figure 5.

$$2\,R_2CHOO^{\bullet} \rightarrow R_2\text{–}CH\text{–}OOOO\text{–}HCR_2 \xrightarrow{-2H} 2\,R_2C{=}O + O_2 \quad [5]$$

$$R_2CHO^{\bullet} + X^{\bullet} \rightarrow R_2C{=}O + XH \quad [6]$$

Disproportionation of alkoxy radical (Eq. 4) would be expected to produce equal amounts of hydroxy and ketodiene compounds by the Russell mechanism (30). However, as discussed by Gardner (26), this pathway was not considered to be important.

In this work, antioxidants were shown to decrease the formation of keto dienes and increase the formation of hydroxy dienes. With Trolox, the ratio of hydroxy to ketodiene compounds was higher than with α-tocopherol. Therefore, hydrogen donating antioxidants can greatly influence the reaction pathways leading to stable hydroxy compounds.

Although the hydrophilic Trolox was only slightly soluble in hexadecane, it was more effective in inhibiting MeLoOOH decomposition than the lipophilic α-tocopherol. This phenomenon has not been previously reported for the decomposition of pure hydroperoxides, but it is consistent with our previous observations that these antioxidants have different effects on hydroperoxide and hexanal formation during autoxidation of corn oil (8). In a bulk corn oil system, even though the hydrophilic antioxidants Trolox and ascorbic acid were not completely soluble, they were more effective than the lipophilic antioxidants α-tocopherol and ascorbyl palmitate. However, in the corresponding corn oil-in-water emulsion system, the lipophilic antioxidants were more effective than their hydrophilic analogues in inhibiting hydroperoxide formation and decomposition. In emulsions, the higher protection of lipophilic antioxidants was explained by their higher affinity toward the oil–water interface. In bulk oils, the better protection of hydrophilic antioxidants was explained by their preferential orientation in the air–oil interface. This interfacial phenomenon supports our finding that the hydrophilic Trolox is more effective than the lipophilic α-tocopherol in inhibiting hydroperoxide decomposition in the nonpolar hexadecane solution. In our previous study (8), we also observed that α-tocopherol was more effective in inhibiting hydroperoxide formation at 232 than at 1161 μM, but the reverse trend was observed in inhibiting hydroperoxide decomposition based on hexanal determination. These results

support our previous study (6), showing that α-tocopherol is an effective inhibitor of the β-cleavage (Eq. 1) responsible for hexanal formation.

This study was limited to the formation of three groups of decomposition products formed at 60°C—hexanal, hydroxy, and ketodiene compounds, which can be formed *via* alkoxyl radicals by β-scission (Eq. 1), hydrogen abstraction (Eq. 2), and hydrogen elimination (Eq. 6). However, other reactions of lipid alkoxyl radicals may be significant under different conditions (25). During Fe(II)-induced decomposition of the 13-hydroperoxide of linoleic acid in methanol solution in the presence of a vitamin E model compound (2,2,5,7,8-pentamethyl chromanol-6-ol), under either aerobic or anaerobic conditions, the formation of epoxy compounds through epoxyallylic radicals, formed by rearrangement of alkoxyl radicals, was suggested as a possible pathway of decomposition (31). In another study (32), the decomposition of 13-MeLoOOH in air in the presence of equal molar concentrations of Fe(III) acetylacetonate and α-tocopherol in benzene solution at 37°C, produced mixtures of peroxy-α-tocopherones, epoxy-hydroperoxy-α-tocopherones, and tocopherol dimers. Under these conditions, the formation of both peroxyl and alkoxyl radicals, rearranging into allylic epoxy-hydroperoxyl radical intermediates, may account for the lipid–tocopherol adducts identified. Further research is under way to clarify the effect of antioxidants on the formation of other nonvolatile compounds, including polymeric products and epoxides, from the thermal decomposition of lipid hydroperoxides.

ACKNOWLEDGMENT

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Physicochemical Characterization of ATP Binding to Human 5-Lipoxygenase

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ABSTRACT: Human 5-lipoxygenase requires ATP as a stimulatory factor. At the two preferred concentrations of the free Ca^{2+}, 0.02 μM with a resting cell and 20 μM with a stimulated cell, Scatchard analysis revealed that 5-lipoxygenase has one affinity ATP binding site with a K_d of 4.6 μM at the low Ca^{2+} concentration but has two affinity ATP binding sites with a higher K_d of 4.4 μM and a lower K_d of 14.5 μM at the high Ca^{2+} concentration. In contrast, in a Tween 20 reaction system, 5-lipoxygenase had similar activation coefficients for ATP at both Ca^{2+} concentrations; these were 12.7 μM at the low Ca^{2+} concentration and 12.0 μM at the high Ca^{2+} concentration. These results showed that 5-lipoxygenase has an ATP binding site and suggest that self-association of 5-lipoxygenase in 20 μM Ca^{2+} may affect ATP binding affinity as measured by Scatchard analysis.

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5-Lipoxygenase (EC 1.13.11.34) is responsible for the first two steps in the formation of leukotrienes, which exhibit biological activities related to inflammatory and allergic responses (1,2). These two steps are the oxygenation of free arachidonate to form 5*S*-hydroperoxy-6-*trans*-8,11,14-*cis*-icosatetraenoic acid (5-HPETE) by 5-HPETE synthetase activity and the subsequent dehydration of 5-HPETE to form 5,6-*trans*-oxido-7,9-*trans*-11,14-*cis*-icosatetraenoic acid (LTA_4) by LTA_4 synthetase activity. LTA_4 is further converted to 5*S*,12*R*-dihydroxy-6,14-*cis*-8,10-*trans*-icosatetraenoic acid (LTB_4), a chemotactic factor, and to the peptidoleukotrienes known as the slow-reactant substance A. Thus, the production of LTA_4, initiated by 5-lipoxygenase, is the first step in the synthesis of leukotrienes, which are of great significance in the pathophysiology of inflammation and anaphylaxis.

5-Lipoxygenase has been purified from several sources such as human leukocytes (3,4), porcine leukocytes (5), rat basophilic leukemia cells (6,7), and mouse mastocytoma cells (8). The enzyme activity is modulated by calcium (9), ATP, and detergents (3,4,8,10). Experiments with partially purified enzyme from guinea pig neutrophils showed that ATP stimulates 5-lipoxygenase activity more than GTP, UTP, AMP, and cAMP (11). 5-Lipoxygenase cDNA has been isolated from human placenta (12), dimethyl sulfoxide-differentiated HL-60 cells, and rat basophilic leukemia cells (13). Although these sequence studies of the enzyme could not reveal any known ATP binding sequence motif in the enzyme, ATP-sepharose affinity chromatography was first applied to purify 5-lipoxygenase from rat basophilic leukemia cells (14), and a successful purification of the enzyme by this method was reported recently for a recombinant enzyme (15). This suggests that 5-lipoxygenase may have an ATP binding site(s). However, it is still unknown how many ATP molecules bind to 5-lipoxygenase and how its binding affinity is. In this report, we describe the stimulatory effects of ATP on purified human 5-lipoxygenase.

MATERIALS AND METHODS

Materials. Arachidonate (>99%) was purchased from Caymen (Ann Arbor, MI) and purified with the CC-4 column method described elsewhere (4). The resulting solution containing arachidonate was concentrated under argon gas. The concentrated arachidonate was diluted with ethanol in which oxygen was substituted with argon gas to avoid air oxidization. Polyoxyethylene sorbitanmonolaurate (Tween 20) containing less hydroperoxide, adenosine 5′-(α-thio)triphosphate (α-S-ATP) and adenosine 5′-(γ-thio)triphosphate (γ-S-ATP) were from Calbiochem (San Diego, CA). Other nucleotides were from Sigma (St. Louis, MO). Inorganic triphosphate was a gift from Professor Junichiro Mukai of the Graduate School of Genetic Resources Technology, Kyushu University. [γ-^{32}P]-ATP (110 TBq/mmol) was obtained from Amersham (Arlington Heights, IL). Other reagents were of the highest grade available.

Enzyme purification. Human recombinant 5-lipoxygenase was expressed and purified as described previously (10).

Enzyme assays by spectrophotometrical and continuous monitoring. The standard assay for 5-HPETE synthetase/LTA_4 synthetase activities in the purified enzyme was performed in

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Abbreviations: α-S-ATP, adenosine 5′-(α-thio) triphosphate; γ-S-ATP, adenosine 5′-(γ-thio) triphosphate; 5-HPETE, 5*S*-hydroperoxy-6-*trans*-8,11,14-*cis*-icosatetraenoic acid; LTA_4, 5,6-*trans*-oxido-7,9-*trans*-11,14-*cis*-icosatetraenoic acid; LTB_4, 5*S*,12*R*-dihydroxy-6,14-*cis*-8,10-*trans*-icosatetraenoic acid; NTA, nitrilotriacetic acid; Tween 20, polyoxyethylene sorbitanmonolaurate.

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0.5 mL of a 0.1 M HEPES buffer (pH 8.0) containing Ca^{2+}-nitriotriacetic acid (NTA) buffer, various concentrations of ATP, 0.5 mM DTT, 0.1 mM arachidonate, and 0.01% Tween 20. Arachidonate-Tween 20 mixed micelles were added to the reaction mixture after arachidonate was vigorously mixed with Tween 20.

After 1 min of preincubation in cuvettes (1 cm light path) at 30°C, the reaction was initiated by adding 6 μg of the enzyme. The progress of the reaction was followed spectrophotometrically by monitoring the absorbance at 236 nm for 5-HPETE and at 280 nm for the two LTB_4 isomers derived from the nonenzymatic degradation of LTA_4 using a Shimadzu (Tokyo, Japan) spectrophotometer model UV-2200. Molar absorption coefficients of 23 mM^{-1} cm^{-1} (16) at 236 nm for 5-HPETE and 39.5 mM^{-1} cm^{-1} (17) at 280 nm for the two LTB_4 isomers were used.

5-Lipoxygenase has two activities, the initial synthesis of 5-HPETE and the subsequent conversion to LTA_4. Therefore, we assumed the true velocity of 5-HPETE to be the summation of the 5-HPETE and LTA_4 velocities.

Tween 20 was used in this study because, as shown in previous studies, a clear reaction mixture could be obtained and a higher 5-HPETE synthetase activity could be observed with Tween 20 than with the other detergents (10). We prepared a fresh Tween 20 solution for each experiment to avoid peroxidation.

Binding assay. 5-Lipoxygenase (6 μg) was mixed with [γ-^{32}P]-ATP in 0.1 M HEPES buffer (pH 8.0) containing Ca^{2+}-NTA buffer, 0.5 mM DTT and incubated at 30°C for 5 min in a final volume of 100 μL. The incubation was terminated by the addition of 20 μL of 0.5% gamma globulin and 250 μL of ice-cold 30% polyethylene glycol 6000. This mixture was centrifuged for 5 min at 15,000 × *g*, 4°C. The radioactivity of the precipitate was measured by liquid scintillation spectrometry. Nonspecific binding was measured in the presence of 100 mM ATP.

Calculation of free calcium concentrations. The free Ca^{2+} concentration of the calcium ion buffer was calculated according to the formula that uses a pK_d of 6.5 for calcium-NTA at pH 8.0 (18).

Miscellaneous. Protein concentrations were determined by the Bradford method using a Bio-Rad Protein Assay kit (Richmond, CA) with bovine serum albumin as the standard. The protein concentration of the purified 5-lipoxygenase was determined by using a molar extinction coefficient of 1.2 × 10^5 M^{-1} cm^{-1} at 280 nm (12,13).

All experiments were repeated at least three times and all data are shown as the average values of the experiments.

RESULTS

Activation coefficients of ATP to the 5-HPETE activity. The effects of ATP on two representative Ca^{2+} concentrations were investigated. In our previous paper (10), little activity was detected at less than 0.1 μM Ca^{2+}; 5-lipoxygenase and LTA_4 synthetase activities attained their maxima over 5 μM Ca^{2+} as determined by the binding of the enzyme to phosphatidylcholine liposomes (19). Based on those results, we established Ca^{2+} concentrations of 0.02 and 20 μM as our representative low and high concentrations for determining the effects of ATP under different free Ca^{2+} concentrations in the Ca^{2+}-NTA system (18). As shown in Figure 1, the addition of ATP to 0.02 μM Ca^{2+} achieved a ninefold increase of 5-HPETE synthetase activity, whereas the addition of ATP to 20 μM Ca^{2+} caused only a threefold increase of activity. The maximum activity of the enzyme at 0.02 μM Ca^{2+} was approximately 80% of the maximum activity at 20 μM Ca^{2+}. Regardless of the Ca^{2+} concentration, the activation coefficients of 5-HPETE synthetase in the presence of ATP were similar when the activation coefficients were calculated from a double-reciprocal plot of the steady-state velocity (K_a = 12.0 μM at the high Ca^{2+} concentration and 12.7 μM at the lower).

Binding of ATP to 5-lipoxygenase. To determine how many ATP molecules bind to a 5-lipoxygenase molecule, the

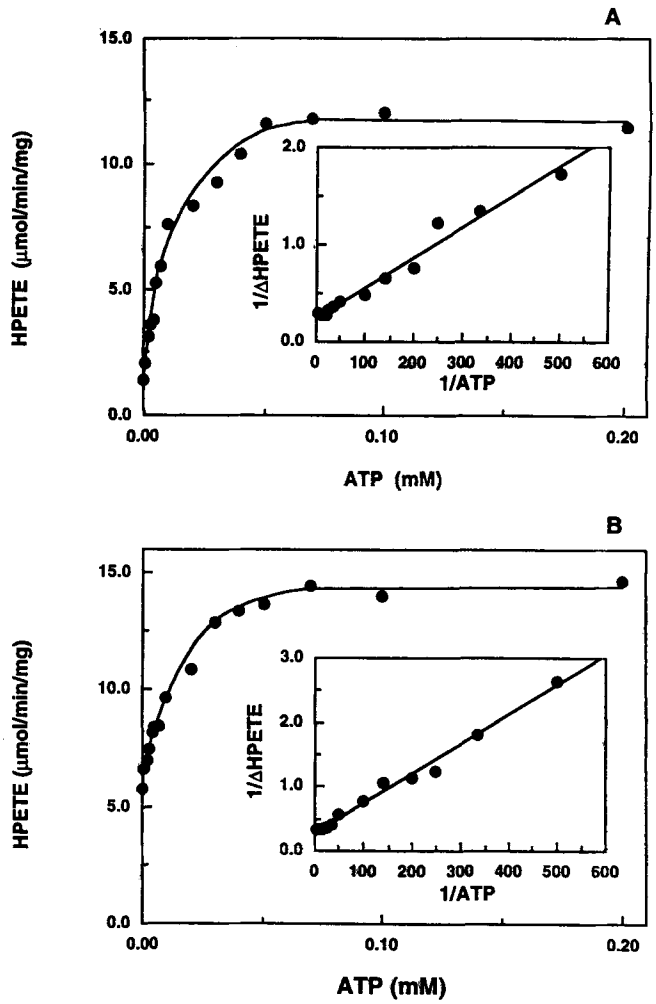


FIG.1. Enhancement of 5*S*-hydroperoxy-6-*trans*-8,11,14-*cis*-isosatetraenoic acid (5-HPETE) synthetase activity by ATP concentration at 0.02 (A) and 20 μM (B) free Ca^{2+}. Free Ca^{2+} concentrations were controlled by the calcium ion buffer. The velocities were fitted to the double-reciprocal analysis.

purified 5-lipoxygenase was subjected to Scatchard analysis of [γ-^{32}P]-ATP binding to the enzyme without any detergent. Scatchard analysis indicated one ATP binding site per 5-lipoxygenase molecule at either the low or the high Ca^{2+} concentration (n = 1.0 at 0.02 μM Ca^{2+} and n = 1.2 at 20 μM Ca^{2+}, Fig. 2). A simple ATP binding with a K_d of 4.6 μM was observed at the low Ca^{2+} concentration. On the other hand, the two different slopes at the high Ca^{2+} concentration showed that 5-lipoxygenase had two affinity sites for ATP with a higher K_d value of 4.4 μM (n = 0.6) and a lower K_d value of 14.5 μM (n = 0.6).

ATP requirement of 5-HPETE synthetase activity and effects of other nucleotides on 5-HPETE synthetase activity. Among the tested nucleotides, γ-S-ATP most effectivly stimulated the activity of 5-HPETE synthetase (Table 1). In this reaction system, α-S-ATP was somewhat less effective than ATP, the effect of dATP was approximately 60% of ATP, and ADP and AMP were less effective than dATP. Other triphosphate nucleotides, adenosine, adenine, and inorganic pyrophosphate, seemed to have no stimulative or inhibitory effect on 5-HPETE synthetase activity. Previously, we found that phosphatidylcholine liposomes increased the conversion ratio of 5-HPETE to LTA_4 (19); however, in this study, none of the nucleotides tested were found to enhance LTA_4 synthetase activity of the enzyme.

TABLE 1
Effects of Various ATP Analogues on 5-HPETE Synthetase Activity[a]

Additions	Enzyme activity (μmol/min/mg)	Relative activity (%)
None	2.65	100.0
ATP	12.80	483.2
ADP	6.15	232.2
AMP	3.71	140.0
cAMP	3.24	122.2
α-S-ATP	11.00	415.1
γ-S-ATP	15.99	603.6
ITP	3.66	138.1
GTP	2.62	98.9
CTP	3.41	128.9
dATP	8.04	303.4

[a]Enzyme was incubated in the assay mixture containing 80 μM Ca^{2+}, 100 μM arachidonate, and 0.01% Tween 20 with various nucleotides at 0.1 mM. Enzyme activities are expressed as initial velocities. 5-HPETE, 5*S*-hydroperoxy-6-*trans*-8,11,14-*cis*-isosatetraenoic acid.

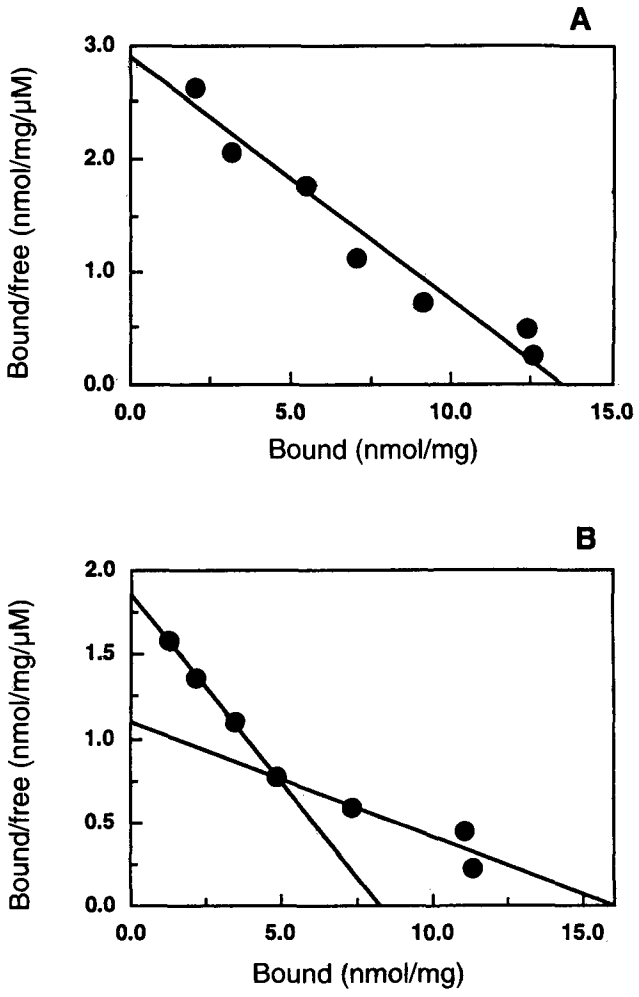


FIG. 2. Scatchard analysis of [γ-^{32}P]-ATP binding to purified 5-lipoxygenase at 0.02 μM (A) and 20 μM (B) free Ca^{2+}. Free Ca^{2+} concentrations were controlled by the calcium ion buffer.

DISCUSSION

Kinetic analysis showed that in the presence of Tween 20 and 0.02 μM Ca^{2+}, ATP enhances 5-HPETE synthetase activity with Ka = 12.7 μM and that in the presence of Tween 20 and a higher Ca^{2+} concentration of 20 μM, ATP enhances 5-HPETE synthetase activity with K_a = 12.0 μM; the 5-lipoxygenase activation coefficients for ATP at the different Ca^{2+} concentrations were similar (Fig. 1). In the absence of Tween 20, however, Scatchard analysis revealed a single binding site of 5-lipoxygenase with a K_d of 4.6 μM at the low Ca^{2+} concentration, and two binding sites with K_d of 4.4 and 14.5 μM at the high Ca^{2+} concentration (Fig. 2); this indicated that in the absence of Tween 20 the binding mode of ATP to 5-lipoxygenase at the low Ca^{2+} concentration differed from the binding at the high Ca^{2+} concentration. In the presence of high Ca^{2+}, 5-lipoxygenase translocates to phosphatidylcholine liposomes due to a conformational change (19). In the absence of detergent, the conformation of the enzyme changes as it binds Ca^{2+} and reveals a hydrophobic region that may allow it to self-associate through hydrophobic interactions. Tween 20 is used extensively to solubilize membrane-associated proteins while maintaining their activities. When Tween 20 is present, after Ca^{2+} binds 5-lipoxygenase, the detergent may cover the newly exposed hydrophobic portion of the enzyme that might otherwise interact with phosphatidylcholine liposomes or with other 5-lipoxygenase molecules. This model is consistent with the similar K_a values we obtained irrespective of Ca^{2+} concentration in the enzyme assay with Tween 20 (Fig. 1). On the other hand, our binding assay system did not contain phosphatidylcholine liposomes, to which 5-lipoxygenase translocates when it is activated at the

TABLE 2
Effects of Adenosine, Inorganic Pyrophosphate (PPi), and Inorganic Triphosphate (PPPi) on 5-HPETE Synthetase Activity[a]

Additions	Enzyme activity (μmol/min/mg)	Relative activity (%)
None	2.65	100.0
Adenosine	3.53	133.3
PPi	3.33	125.5
PPi	3.52	132.9
Adenosine + PPi	4.02	151.6
Adenosine + PPPi	2.79	105.4

[a]Enzyme was incubated in the assay mixture containing 80 μM Ca^{2+}, 100 μM arachidonate, and 0.01% Tween 20 with adenosine, PPi, and PPPi at 0.1 mM. Enzyme activities are expressed as initial velocities. Abbreviation as in Table 1.

high Ca^{2+} concentration, thus the binding affinity of ATP to 5-lipoxygenase should be lower in our system because of enzyme self-association in the absence of detergents. One interpretation of these results is that a 5-lipoxygenase molecule may have an ATP binding site with a binding affinity to ATP that is constant, irrespective of the presence of Ca^{2+}.

The value of K_d at the low Ca^{2+} concentration and the value of K_a for ATP differed (Figs. 1 and 2). Tween 20 may affect the conformation of the enzyme so that the value of K_a for ATP is much lower than what might be predicted by the K_d of the binding assay. Such a conformational change of the enzyme also may occur in the absence of Tween 20 when 5-lipoxygenase molecules associate with each other at the high Ca^{2+} concentration, and may account for the lower K_d comparable to the K_a value for ATP.

It has been reported that ATP stimulated the 5-lipoxygenase activities more than the other ATP analogues (11). In this report, we have shown that γ-S-ATP stimulated 5-HPETE synthetase activity more than ATP (Table 1). α-S-ATP resulted in less activity than ATP. These results showed that the γ-phosphate moiety of ATP is important for the stimulation. Because ADP and the other nucleotides with triphosphate were less effective than ATP, the charges on the adenine and γ-phosphate moieties may cooperatively affect the enzyme activity. dATP affected ~60% of ATP on 5-HPETE synthetase enhancement. Differences in the ribose moiety of these nucleotides affected the activity of the enzyme less than the difference in the base. The ribose ring may work as a spacer which arranges the relative positions of base and triphosphate. The mixture of both adenosine and these free inorganic phosphates did not enhance the enzyme activity as significantly as the addition of adenosine alone (Table 2), therefore, the conformation of both the adenine-base and the phosphate moieties appears to be essential for the activation of 5-lipoxygenase. Unexpectedly, the addition of adenosine and inorganic pyrophosphate to the reaction mixture showed more effective enhancement than the addition of adenosine and inorganic triphosphate. We obtained the same results as above on LTA_4 synthetase (data not shown).

Finally, 5-lipoxygenase did not show any ATP-hydrate activity or any self-phospholylation (data not shown). These results were consistent with stimulations of the enzyme activity by γ-S-ATP and α-S-ATP; thus, it is concluded that ATP, like Ca^{2+}, acts as a co-factor of 5-lipoxygenase activity.

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Sterol Specificity of the *Saccharomyces cerevisiae ERG6* Gene Product Expressed in *Escherichia coli*

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ABSTRACT: The *ERG6* gene from *Saccharomyces cerevisiae* has been functionally expressed in *Escherichia coli*, for the first time, yielding a protein that catalyzes the bisubstrate transfer reaction whereby the reactive methyl group from (*S*)-adenosyl-L-methionine is transferred stereoselectively to C-24 of the sterol side chain. The structural requirements of sterol in binding and catalysis were similar to the native protein from *S. cerevisiae*. Inhibition of biomethylation was observed with fecosterol and ergosterol which suggests that ergosterol may function in wild-type yeast as a feedback regulator of sterol biosynthesis.
Lipids 31, 373–377 (1996).

The (*S*)-adenosyl-L-methionine:$\Delta^{24(25)}$-sterol methyl transferase (SMT: EC 2.1.1.41) synthesized by *Saccharomyces cerevisiae* catalyzes the stereoselective transfer of the (*S*)-adenosyl-L-methionine (AdoMet) methyl group to the 24,25-double bond of the sterol side chain (Fig. 1). The crucial role of this enzyme in regulating production of ergosterol, which functions as the fungal membrane insert, has stimulated considerable interest in the kinetic properties, stereochemistry, and mechanism of the transfer reaction (1–4), and in the design of inhibitors of SMT activity which may be used clinically or agrochemically as antifungal agents (5,6). The putative gene encoding the SMT from *S. cerevisiae* has been cloned and sequenced (7–9); however, there is no enzymological evidence to demonstrate that *ERG6* encodes an SMT enzyme. The purpose of this communication is to report for the first time enzymological evidence that *ERG6* is the yeast SMT structural gene and the sterol substrate specificity of the gene product.

MATERIALS AND METHODS

Media, strains, and plasmids. Escherichia coli AG-1 cells were used in transformation experiments. Plasmid pBR322 (10) was used as the cloning vector. Plasmid pRG458 (7) was provided by Dr. Martin Bard (Dept. of Biology, Indiana University, Indianapolis, IN) and used as the source of *ERG6* DNA. Cells were routinely maintained on Luria broth medium (11). Lucia broth medium was supplemented with ampicillin at a final concentration of 50 μg/mL when culturing cells that contained plasmid DNA. Cells were made competent using a solution of 50 mM $CaCl_2$ (11). SOB medium (12) was used for cell recovery following transformation with plasmid DNA.

Subcloning and DNA sequence analysis. DNA fragments generated by digestion of pRG458 with *Dra* I and *Nhe* I to isolate the open reading frame (ORF) were separated by agarose gel electrophoresis. The 1281 base pair fragment was eluted from the gel, extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), precipitated in ethanol, dried, and dissolved in *tris*-EDTA buffer (11). The DNA was made blunt-ended by incubation with T4 DNA polymerase and deoxynucleoside triphosphates and ligated into *Eco*R V-linearized, dephosphorylated pBR322. Protocols for plasmid and DNA fragment isolation, restriction endonuclease digestion, ligation, and transformation were either standard (11) or were those recommended by the manufacturer. Shrimp alkaline phosphatase was purchased from Ambion (Austin, TX). Restriction enzymes were purchased from U.S. Biochemicals (Amersham, Arlington Heights, IL) and Promega Corporation (Gaithersburg, MA). Reagents for the preparation of blunt-ended *ERG6* fragment and ligation reactions were purchased as kits from Panvera Corporation (Madison, WI). Dideoxynucleotide sequencing of plasmid DNA clones was performed by the chain termination method (13) using the reagents and protocol from a Sequenase II kit purchased from U.S. Biochemicals. Oligonucleotides used to prime the sequencing reactions were synthesized at the Texas Tech University Biotechnology Institute Core Facility (Lubbock, TX).

Expression of SMT activity and measurement of enzyme kinetics. Escherichia coli cells that contained pSMTy23 were cultured at 37°C to a culture A_{600} of 0.8 to 1.0. The cells were harvested by centrifugation (4,000 × *g* for 10 min). The resulting pellet was resuspended in 5 mL of buffer (50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 2 mM 2-mercaptoethanol) and ruptured using a French Press (SLM Instruments, Urbana, IL). The cell debris was removed by centrifugation (12,000 × *g* for 15 min), and the supernatant fraction (batch enzyme) was used as the source of material for the SMT assay system (14). SMT assays were performed on 0.5 mL batch enzyme in reactions that contained 50 μM zymosterol and 50 μM [*methyl*-$^3H_3$]AdoMet as described elsewhere with constant agitation at 55 rpm on a shaker bath at 30°C for 45 min

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Abbreviations: AdoMet, (*S*)-adenosyl-L-methionine; HPLC, high-performance liquid chromatography; ORF, open reading frame; SMT, (*S*)-adenosyl-L-methionine:$\Delta^{24(25)}$-sterol methyl transferase.

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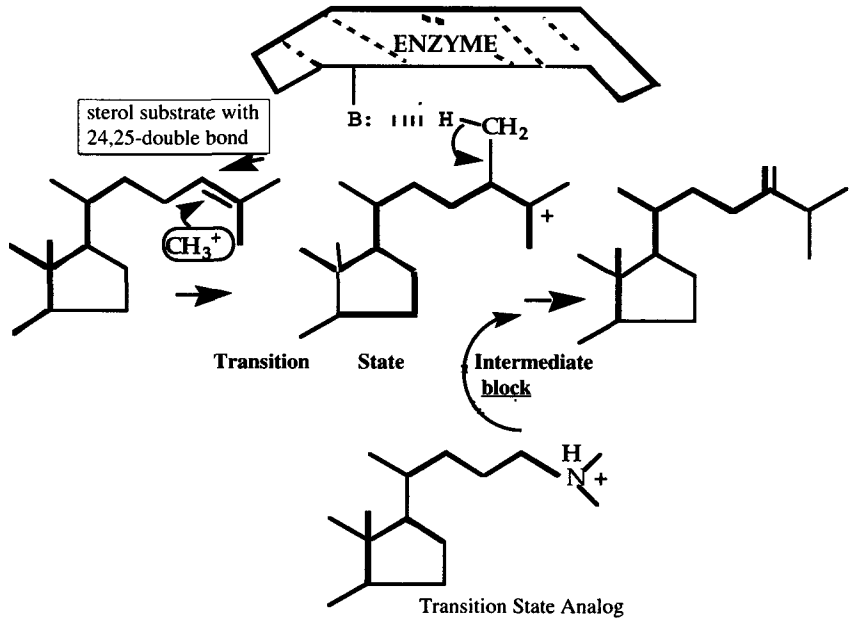


FIG. 1. Mechanism of the (*S*)-adenosyl-L-methionine: $\Delta^{24(25)}$-sterol methyl transferase-catalyzed transmethylation of sterol and (*S*)-adenosyl-L-methionine to yield a 24(28)-methylene sterol. The sterol nomenclature system used in this paper follows the *Nes* rules discussed in Reference 19 and references cited therein. Thus the exocyclic methylene carbons are numbered C-24-C-28, not 24(24').

(15,16). Reactions were terminated by adding 10% methanolic KOH. Nonsaponifiable lipids were extracted from the reaction mixtures with diethyl ether and the organic-soluble sterols and radioactivity analyzed as described (15,16). Inhibitor studies were performed using batch enzyme in the presence of 5 to 100 μM zymosterol and 50 μM [*methyl*-$^3H_3$]AdoMet, and inhibitor concentrations were tested over the range of 1 nM to 100 μM. Inhibition type and K_i values were determined by standard graphical procedures (15,16) for which computer-assisted linear regression analysis afforded

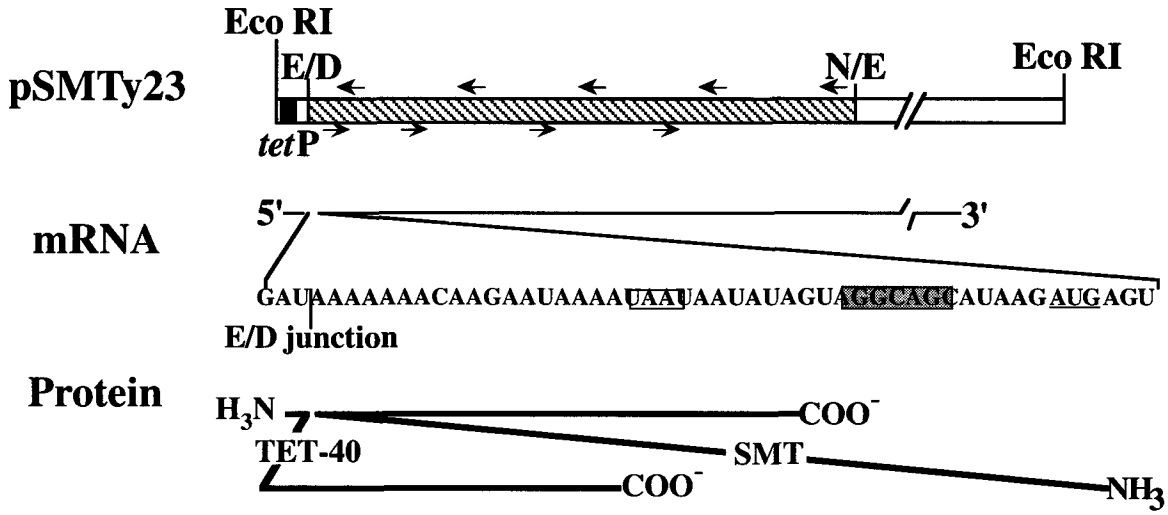


FIG. 2. Cloning, sequencing, and organization of pSMTy23; pSMTy23 DNA is shown as an *Eco*R I linearized plasmid. The *Eco*R V/*Dra* I (E/D) and *Nhe* I/*Eco*R V (N/E) junctions flank the DNA, derived from pRG458, that contains the *ERG6* structural gene (hatched region). The approximate positions and orientation of the oligonucleotide primers used to sequence the pSMTy23 insert are represented by arrows. The pBR322 tetracycline resistance promoter (*tet*P) is represented as a black box. The mRNA that originates from *tet*P has an open reading frame that encodes a truncated (40 amino acid, TET-40) form of the tetracycline resistance gene product whose translation terminates as the result of an in-frame UAA (boxed) located in the cloned DNA fragment. A purine-rich sequence (shaded box), which may serve as a ribosome binding site for (*S*)-adenosyl-L-methionine: $\Delta^{24(25)}$-sterol methyl transferase (SMT) translation, is located six bases upstream of the putative SMT initiator codon (underlined).

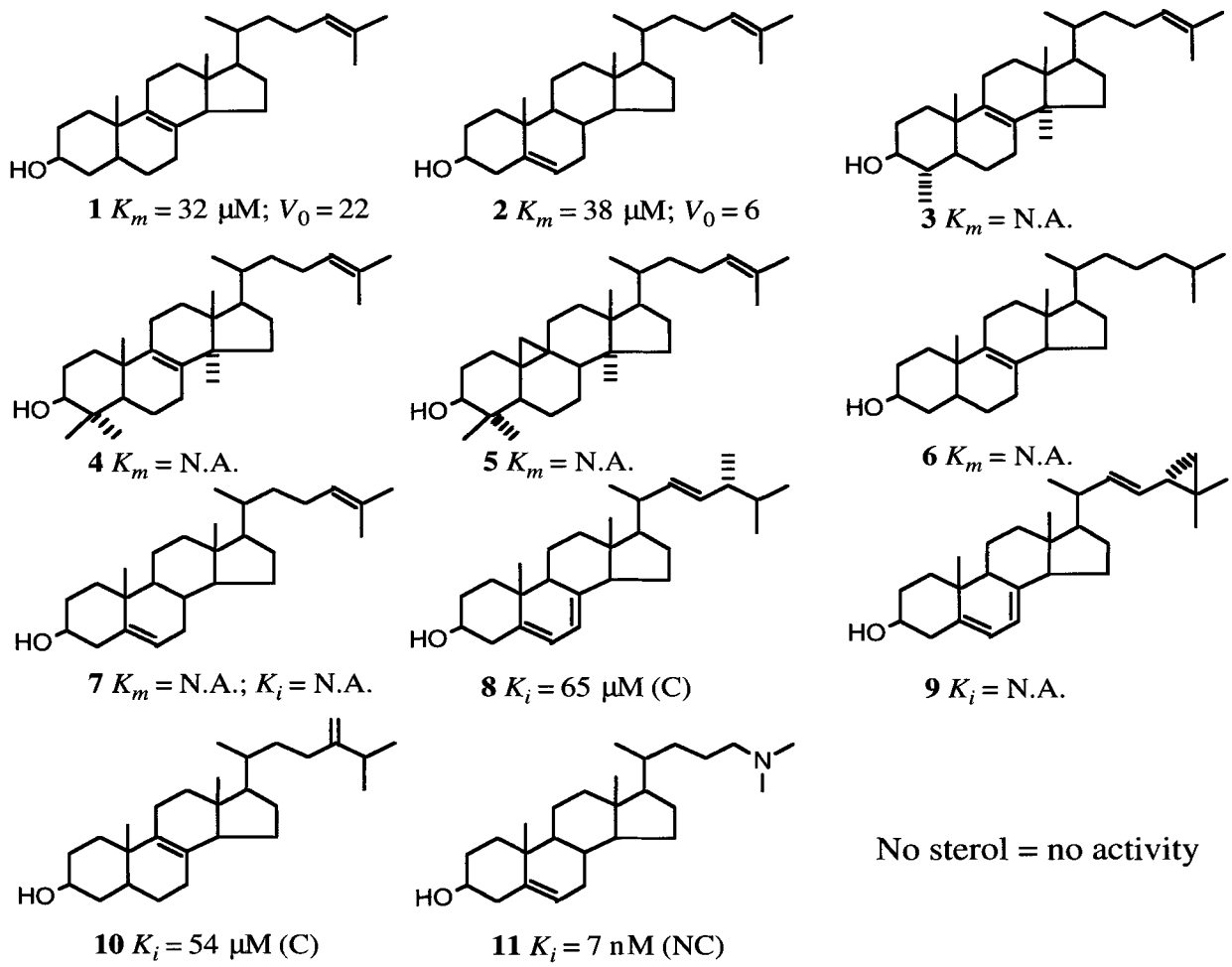


FIG. 3. Sterol substrate specificity and inhibitors of (*S*)-adenosyl-L-methionine: $\Delta^{24(25)}$-sterol methyl transferase enzyme expressed in *Escherichia coli.*

correlation coefficients greater than 0.98 in all trials (n = 3). Products of the SMT reactions were analyzed by high-performance liquid chromatography (HPLC)-radiocounting and gas chromatography/mass spectrometry (GC/MS) (15,16). Protein was determined as described (17). Source of compounds was described elsewhere (15,16; Nes, W.D., and Guo, D., unpublished data). Attempts to overexpress the *ERG6* gene in *E. coli* using plasmid vector, e.g., pLEX (P_L expression system from Invitrogen, San Diego, CA) were impeded by the nonavailability of a restriction site which was appropriate for cloning.

RESULTS AND DISCUSSION

Our initial efforts were directed to the cloning and sequencing of the putative open reading frame (ORF) of *ERG6*. The purpose of these experiments was to confirm that the *ERG6* DNA used in this study was identical to *SED6*, the gene claimed by Hardwick and Pelham (8) to be *ERG6*. *Escherichia coli* has been found to lack the capacity to synthesize sterols (18). For this reason, *E. coli* was chosen as the expression system of the *ERG6* gene. The *SED6* gene sequence published by Hardwick and Pelham (8) was analyzed using the program GeneJockey (Biosoft) to determine that *Dra* 1 and *Nhe* 1 were appropriate restriction enzymes for subcloning the ORF from the plasmid pRG458. The 1.3 kilobase pair fragment obtained by digesting pRG458 with *Dra* 1 and *Nhe* 1 was subcloned into the *Eco*R V site of pBR322. Two unique clonal types, pSMTy23 and pSMTy10, were obtained (Fig. 2). Restriction enzyme digestion patterns of the plasmids determined that these clones differed in the orientation of the insert. DNA sequence analysis of pSMTy23 confirmed that this clone contained a sequence identical to the putative *ERG6* gene published by Hardwick and Pelham (8).

Batch enzyme preparations from cells that contained either pSMTy23 or pSMTy10 were analyzed for SMT activity as described in the Materials and Methods section. Preparations from cells that contained pSMTy23 were found to contain SMT activity, whereas preparations from cells that contained pSMTy10 did not. Our rationale for the basis of SMT

enzyme expression from pSMTy23 in *E. coli* is that *ERG6* mRNA was synthesized in a transcript which originated at the tetracycline resistance determinant promoter of the vector. The bacterial-expressed recombinant protein from this vector system is likely to give rise to the native protein rather than a fusion protein. A purine-rich sequence located upstream of the putative SMT start codon is predicted to serve as a ribosome binding site for translation (Fig. 2).

The functional importance of precise structural and electronic features of the methyl acceptor molecule in supporting SMT activity was first examined in reactions that contained either cholest-8-enol or cholesta-8,24-dienol (zymosterol) as substrate. Methyl transfer occurred only in those reactions that contained zymosterol, demonstrating the importance of the 24,25-double bond in the sterol side chain for catalysis (Fig. 3). HPLC-radiocounting was performed on Whatman reversed-phase C_{18}-columns designed to separate substrate from 24(28)-methylene product and related 24-alkene isomers (19). As shown in Figure 4, zymosterol was stereoselectively biomethylated to fecosterol. To obtain sufficient quantities of biomethylated product for chemical identification, 20 assays were performed, and approximately 4 μg of chromatographically pure sterol was collected from the HPLC column. The sterol was identifed as fecosterol by comparison of its chromatographic behavior in thin-layer chromatography (R_f 0.3, analytical plates developed twice in 85:15 benzene/ether), HPLC (0.77, α_c on Whatman C_{18}-column (Maidstone, England) eluted with MeCN/i-PrOH (9:1) at 1 mL/min at ambient temperature, and gas–liquid chromatography (1.31, retention time relative to cholesterol on 3% SE-30 packed column operated isothermally at 245°C), and by its fragmentation pattern in mass spectroscopy (*m/z*, M^+ 398 amu and other diagnostic ions at 383, 365, 341, 314, 327, 299, 285, 271, 245), with that of an authentic specimen (19).

Several naturally occurring sterols with the 24,25-double bond were tested as substrates; zymosterol and desmosterol were found to function as methyl acceptors in the SMT reaction, whereas lanosterol, cycloartenol, and 31-norlanosterol did not serve as substrates for SMT (Fig. 3). Greater catalytic competence (measured as V_{max}/K_m, 15) was observed with zymosterol than desmosterol. Sterol specificity for SMT catalysis exhibited by the protein expressed from pSMTy23 was similar to that of the native enzyme of *S. cerevisiae* (20,21). In contrast to the plant SMT (15), the yeast sterol-SMT complex was found to be sensitive to steric interference from the C-4 methyl group(s) (20). The isosteric transition state analog for the biomethylation reaction, 24β,25-cyclopropyl ergosta-5,7,(*E*)22-trienol, was not an inhibitor of SMT activity, due to the modification at C-24. However, the isoelectronic transition state analog, 25-azacholesterol (at which physiological pH is protonated) (16) was a potent inhibitor of SMT activity, results consistent with previous reports that 25-azacholesterol and related side-chain ammonium-containing sterols are potent inhibitors of yeast SMT activity (4,5,21).

The SMT from pSMTy23 exhibited a competitive-type inhibition by fecosterol, which is consistent with kinetics from

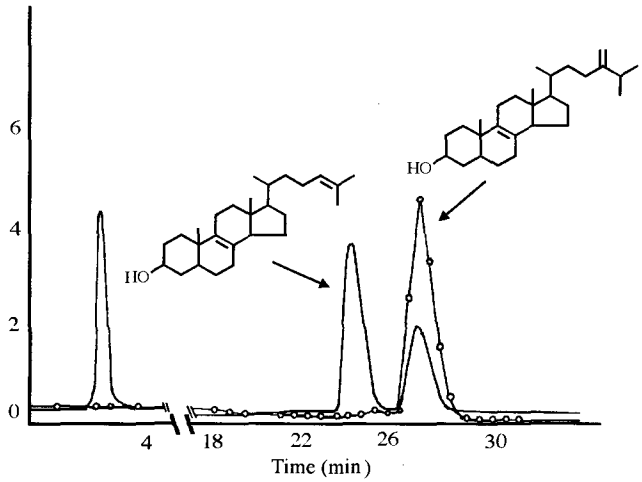


FIG. 4. High-performance liquid chromatography-radiocount chromatogram [Whatman C_{18}-reversed phase column (Maidstone, England) eluted with acetonitrile/isopropanol, 9:1; the ultraviolet detector was set at 205 nm] showing the mass and radioactivity associated with sterols isolated from the thin-layer chromatography-purified nonsaponifiable lipids (NSF) fraction. The NSF was diluted with 50 μg of nonradioactive fecosterol. The sample was obtained from reactions that contained zymosterol and [$^3H_3$-methyl](*S*)-adenosyl-L-methionone as substrates and batch enzyme prepared from pSMTy23-containing cells; O—O, ^3H-radioactivity (dpm x 104); —, detector response (205 nm). Abbreviation as in Figure 2.

product inhibition. When ergosterol and cholesterol were paired as inhibitors, only ergosterol inhibited SMT activity (Fig. 1). This result is expected since cholesterol does not possess a 24-alkyl group. Thus, ergosterol may regulate its synthesis under normal physiological conditions through control of post-lanosterol transformation events as proposed by Moore and Gaylor (20). The inability of others to observe product inhibition by fecosterol (4,5) may be due to the high level of endogenous ergosterol in microsome preparations from wild-type cells (21).

Hydropathy analysis of the SMT enzyme (Fig. 5), based on the predicted amino acid composition from the *ERG6* gene sequence, indicates that the enzyme is moderately hydrophilic with no obvious membrane-localizing elements. For some experiments, batch enzyme was processed by centrifugation at 100,000 × *g* for 90 min to sediment a microsomal fraction (14,22). Microsomal pellets were found to contain 80% of the original SMT activity of the sample, suggesting that the SMT produced in *E. coli* is membrane-associated. Emulphogen-solubilized microsomes released SMT activity with 50% recovery. These observations are consistent with the demonstration that the yeast SMT is loosely bound to the endoplasmic reticulum and distributes into several alternate membrane fractions and into a "floating lipid layer" upon fractionation (14,22).

In this report we demonstrate that the *ERG6* gene expressed from pSMTy23 in *E. coli* produces a protein that mediates biomethylation of zymosterol. The product of the reaction was unambiguously identified as fecosterol using a combination of radiochemical, chromatographic, and spectral

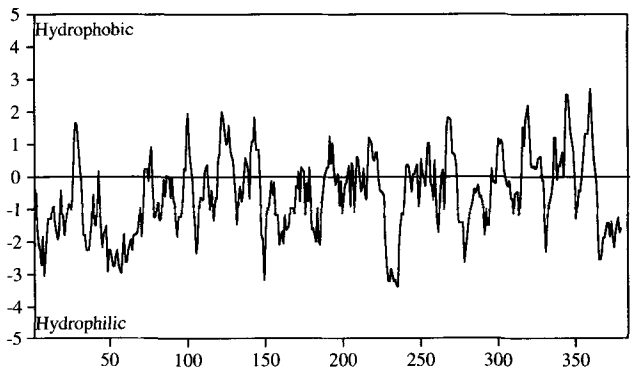


FIG. 5. Hydropathy plot of predicted (*S*)-adenosyl-L-methionine: $\Delta^{24(25)}$-sterol methyl transferase amino acid sequence (Ref. 8).

methods. Down-regulation of the SMT enzyme activity from pSMTy23 by select 24-alkyl sterols shows metabolic specificity for the structure of the side chain. These results confirm that *ERG6* is the structural gene for SMT in yeast and that ergosterol may function to regulate its own synthesis under normal physiological conditions in yeast.

ACKNOWLEDGMENTS

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Purification and Characterization of a *Penicillium* sp. Lipase Which Discriminates Against Diglycerides

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ABSTRACT: A lipase was isolated from *Penicillium* sp. strain UZLM-4 and characterized. This lipase has a molecular weight of 27,344 (determined by mass spectrometry) and hydrolyzes triglycerides in preference to mono- and diglyceride substrates. Among various triglyceride substrates, tributyrin is hydrolyzed about four times faster than any other tested. The lipase has a preference for hydrolysis at the 1,3 positions of the lipids and shows a weak stereoselectivity for the S enantiomer. Unlike most other lipases, this lipase is stable and has a high activity at low surface pressures (5–10 mN/m).
Lipids 31, 379–384 (1996).

Commercial applications of lipases have been investigated for many years. The present and potential uses of lipases span a wide variety of industries, including production of flavor esters for various foods (1), production of surfactants (2), transesterifications for production of various oils, and, more recently, in chiral syntheses (3–5). Among the enzymatic properties which might be important for any given application are the thermal stability, the substrate specificity for both chain length and position, stereoselectivity, and stability in organic solvents.

In addition to the commercial interest in lipases, a great deal of academic interest has been focused on these enzymes. The physical and enzymatic properties of many lipases from a wide variety of sources have been investigated, and in the past few years a number of three-dimensional structures have been reported (6–12). Many microbial lipases including those from *Rhizomucor* (13), *Pseudomonas* (14), *Candida* (15), *Geotrichum* (16,17), *Aspergillus* (18,19), *Rhizopus* (20), and *Penicillium* (21–28) have been isolated and characterized. In this report we describe the purification and characterization of a lipase from *Penicillium* sp. which displays some novel enzymatic properties.

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Abbreviations: HPLC, high-performance liquid chromatography; PDMS, poly(dimethylsiloxane).

MATERIALS AND METHODS

Identification and culture of strain. The strain of *Penicillium* sp. (UZLM-4) was isolated from soil and characterized at the Microbial Enzymes Laboratory of the Uzbekistan Academy of Sciences (Tashkent, Uzbekistan). Screening for the lipase-producing clone of *Penicillium* was done by spreading a suspension of spores on agar-fat plates (a lower layer of animal fat overlaid with 2.0% nutrient agar). The colonies producing the strongest clearing zones were selected. The organism was cultured in 30-L fermenters for 72 h at 25–27°C on a medium containing 5% malt extract, 0.5% sucrose, 0.7% cotton oil, 0.2% $NaNO_3$, 0.1% $CaCO_3$, 0.05% KCl, 0.02% $MgSO_4$, and 0.001% $FeSO_4$ adjusted to pH 5.8–6.0. The cells were removed by filtration. The lipase was found in the culture filtrate, and the crude enzyme preparation was obtained by precipitation with 4 vol of isopropanol. The precipitate was air-dried and stored at −20°C.

Purification of lipase. Ten grams of the crude lipase preparation was redissolved in water (2.5%) and fractionated with acetone. The precipitate obtained at 33–75% acetone (vol/vol) was removed by centrifugation at 3000 × *g* for 20 min at 4°C and was redissolved in 200 mL of 20 mM sodium phosphate buffer, pH 8.5. The enzyme was partially purified batchwise on 50 mL of Q-Sepharose (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer. The material which flowed through the Q-Sepharose in 20 mM buffer without binding was adjusted with saturated ammonium sulfate solution to 10% saturation and applied to a butyl-Sepharose column (Pharmacia) (2.5 × 5 cm) equilibrated with 10% saturated ammonium sulphate. After thoroughly washing unbound material from the column with 10% saturated ammonium sulfate, the enzyme was eluted with a gradient to water. Fractions containing lipase were pooled, concentrated on a YM 3 Amicon membrane (Amicon, Inc., Beverly, MA) (3000 MW cutoff), and diafiltered with 20 mM sodium phosphate buffer, pH 8.5. Concentrated semipurified lipase preparation (10 mL) was applied on a Mono Q FPLC column (1.2 × 10.8 cm) (Pharmacia) equilibrated with 20 mM phosphate buffer, and the enzyme was eluted with a 0–0.2 M NaCl gradient in phosphate buffer,

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TABLE 1
Purification of *Penicillium* sp. UZLM-A Lipase

Purification step	Volume (mL)	Protein[a] (mg/mL)	Lipase[b] (U/mL)	Lipase[b] (U/mg)	Yield (%)
Crude extract	250	12.5	120.7	9.6	100.0
33–75% Acetone fraction	250	9.9	66.2	6.7	54.8
Q-Sepharose (batch)	350	3.6	94.1	26.1	109.1
Butyl-Sepharose[c]	8.5	10.2	3,180.4	311.8	89.6
Mono Q (FPLC)[c]	3.0	2.2	2,202.0	1,000.9	21.9

[a]Protein concentration was determined with modified Lowry method (Ref. 40).
[b]Lipase activity was determined titrimetrically as described in the Materials and Methods section.
[c]Preparation concentrated on YM 3 Amicon membrane (Amicon, Inc., Beverly, MA).

pH 8.5. The summary of the lipase purification is shown in Table 1.

Gel electrophoresis. SDS gel electrophoresis was done on a Phastsystem (Pharmacia, Uppsala, Sweden) using 10–15% gradient gels according to the manufacturer's instructions. Protein bands were stained with Coomassie blue.

Amino acid analysis. Purified protein (50 µg) was hydrolyzed by using the acid vapor technique (27), and the hydrolysate was analyzed on a Beckman System 6300 High Performance Amino Acid Analyzer (Beckman Instruments, Fullerton, CA).

N-*Terminal sequencing. Penicillium* lipase (1–2 µg) was separated on an SDS gel, and the doublet was electroblotted onto a polyvinylidene fluoride membrane (Immobilon; Millipore, Milford, MA) according to the procedure described by Matsudaira (29). The *N*-terminal sequence was determined on an Applied Biosystems (Foster, CA) model 470A gas-phase protein sequenator (30).

Mass spectrometry. The purified protein was dialyzed into 5% acetic acid (vol/vol) for mass spectrometry. The protein mass was determined on an ion spray mass spectrometer (API III Biomolecular Mass Analyzer, Perkin-Elmer Sciex Instruments, Thornhill, Ontario, Canada) calibrated using lysozyme and polyethyleneglycol standards. The molecular weight was calculated from a set of simultaneous equations of the form

$$m/z = \frac{MW + n}{n} \quad [1]$$

where m/z is the measured mass/charge ratio, MW is the molecular weight, and n is the number of protons giving the protein its charge. Successive peaks in the spectrum differ by one proton so that if $m/z(1) < m/z(2)$, then

$$m/z(1) = \frac{MW + n}{n} \quad \text{and} \quad m/z(2) = \frac{MW + n - 1}{n - 1}$$

A series of peaks then provides a set of simultaneous equations from which the value MW can be derived.

Determination of lipase activity. (i) Plate assay. Lipase activity in column fractions was estimated by a rhodamine–triglyceride–agarose plate assay according to the procedure developed in our laboratory (31).

(ii) Titrimetric assay. All lipid substrates were purchased from Nu-Chek-Prep, Inc. (Elysian, MN). Initial velocity was measured using a titrimetric assay according to a recently described procedure (32). An emulsion was prepared by sonicating a mixture of di- or triglyceride (5 mM) and poly(dimethylsiloxane) (PDMS) in 2% (wt/vol) arabic gum solution and 1% $CaCl_2$. The mixture was sonicated for two minutes at 50% power output on a W-375 sonicator (Heat Systems Ultrasonics Inc., Farmingdale, NY). The enzymatic reaction was initiated by addition of 1–10 µg of enzyme to the emulsion. Throughout the assay the pH was maintained at pH 7.0 by titration with 50 mM NaOH by pH-stat (RTS822 Recording Titration System; Radiometer, Copenhagen, Denmark). Lipase activity was expressed in µmoles of fatty acid released per mg of enzyme at 25°C.

(iii) High-performance liquid chromatography (HPLC) measurements. The reaction mixture totalling 5 mL composed of 50 mM phosphate buffer, pH 7.1, and a suspension of 150 mM triolein or diolein was placed in the scintillation vial and stirred with a 12 mm stirring bar at 700 rpm. The 40 µL samples were withdrawn periodically and diluted with 800 µL of acetone, filtered through 0.2 µ HV Millipore filter and the reaction products were analyzed by reverse-phase HPLC (Waters, Milford, MA) on a C18 column (CSC-S ODS2, 25 × 0.46 cm, 5 µm; Supelco, Missassuaga, Ontario, Canada) using acetone/acetonitrile (1:1, vol/vol) as the mobile phase (33). The peak areas of the various reaction products were integrated using the Baseline 810 software (Waters) and compared to respective standards.

(iv) Monolayer measurements. The chloroform solutions of 1,2- or 1,3-dicaprin were spread over 400 cm^2 of 10 mM Tris-HCl, pH 7.0, in 100 mM NaCl containing 22 mM $CaCl_2$ and 1 mM EDTA placed in the "zero-order" trough (34). The surface pressure of the monolayer was controlled by a barostat (KSV Instruments, Helsinki, Finland) equipped with a Wilhelmy plate connected to the electromicrobalance. Lipase (approximately 0.1 µg) was injected under the monolayer film in the "enzyme" compartment of the trough, and the progress of the reaction was monitored by the data station equipped with the software provided by the barostat's manufacturer. Enzyme activity was expressed in µmoles of substrate hydrolyzed per µmol of lipase per minute per cm^2 of the interface at 25°C [µmoles $(DC_{10}) \cdot cm^{-2} \cdot min^{-1} \cdot \mu mol^{-1}$ (E)].

RESULTS

Purification. The isopropanol extraction of the culture filtrate typically gave a twofold purification of the lipase with about 80% recovery of the lipase activity in the resulting dried powder. Table 1 shows the results of a typical purification of the lipase from this crude dried powder. The yield of enzyme purification was typically 20–30% of the activity present in the crude enzyme preparation. The acetone precipitation produced two fractions with lipase activity. The major activity

was purified further to the lipase described in this paper and will be referred to as UZLM-A. No attempts have been made to pursue the other activity because of the low yield. The substrate profile of the second lipase fraction was somewhat different than that of the major fraction, but since we were unable to purify the second fraction to homogeneity, no extensive characterization and comparison were possible.

The acetone precipitation resulted in a loss of some lipase activity and a drop in the specific activity (Table 1). This rather harsh treatment of the enzyme was nonetheless necessary, as with no acetone precipitation the recovery of the lipase in subsequent chromatographic steps was drastically reduced. This parallels results obtained with other lipases produced by fermentation from media containing high concentrations of lipids (Ziomek, E., unpublished results). Although we have no direct evidence of lipid binding to the lipase, we suspect that complexation with lipids or lipopolysaccharides alters the chromatographic behavior of the enzymes and makes recovery difficult. The acetone treatment solubilizes the vast majority of lipids from the mixture and separates the enzyme from the lipids. The Q-Sepharose purification step resulted in a large increase in activity and a recovery of more than 100% (see Table 1). This indicates that at this stage of the purification the enzyme was separated from some inhibitory factors which have not been identified.

Biochemical characterization The MW of the purified lipase estimated from SDS gels is ~28,000. Immediately following purification, a doublet with estimated MW of 28,000 and 26,000 was observed on the SDS gels. The doublet was blotted from the gel and *N*-terminal sequencing of the first 22 residues showed a single strong signal, indicating that the two bands of the doublet must be either identical or closely related proteins. The mass of the enzyme measured by ion spray mass spectrometry is 27,334 ± 1.2 daltons, consistent with the MW estimated from SDS gels (Fig. 1). Small peaks in the mass spectrum suggest the presence of components of about 25,000 and 26,000 MW, consistent with the appearance of the doublet on SDS gels. Since only one sequence was observed from the doublet, these components must be either trace amounts of C-terminally truncated forms of the major lipase activity or closely related lipases. Upon storage of the enzyme preparation at 4°C, some precipitate was observed. After centrifugation to remove the precipitate, a single band at 28,000 MW was observed on SDS gels. No further characterization of the precipitate was done, but the lipase remaining in solution appeared to be homogeneous.

TABLE 2
Comparison of *N*-Terminal Sequences of Some *Penicillium* Lipases[a]

Microbial source	*N*-terminal sequence	Reference
P. sp. UZLM-4	ATAATAAFPDLNXAAKLXXAAY--	—
P. expansum	AVAASAAFPDLXRAAKLSSAVY--	28
P. camembertii	DVSTSELDQFEFWVQYAAASYY--	36

[a]X = unidentified residue.

Comparison of the *N*-terminal sequence shown in Table 2 with those of other known lipase sequences reveals striking similarity to the *N*-terminal sequence of a lipase from *P. expansum* (28). A search of the SWISS-PROT database (35) revealed no other significant sequence similarities. There is weak similarity of the N-terminal sequences of UZLM-A and *P. expansum* lipase to residues 149-170 of *P. camembertii* lipase (28,36). Within this 22-residue stretch, there are two "identities" spanning three residues and one of two residues. However, one must introduce insertions or deletions to align these three "identical" stretches simultaneously. This suggests that the similarities of the UZLM-A and *P. expansum* sequences with this region of *P. camembertii* are fortuitous and of no functional significance.

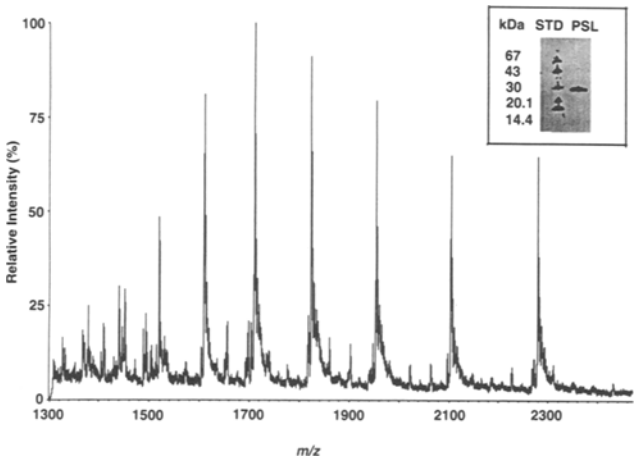


FIG. 1. Mass spectrometric analysis of the purified UZLM-A lipase. The mass calculated from the series of major peaks in this spectrum is 27,344 daltons. The inset shows the SDS–polyacrylamide gel electrophoresis of *Penicillium* sp. UZLM-A lipase. Lane 1, Bio-Rad (Bio-Rad Labs, Richmond, CA) low molecular weight standards. Lane 2, purified *Penicillium* sp. UZLM-A lipase, 0.5 μg.

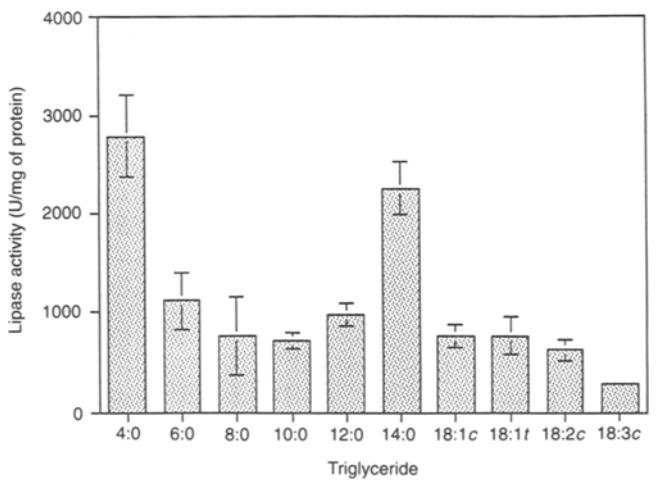


FIG. 2. Substrate profile of *Penicillium* sp. UZLM-A lipase. Activities were measured using the titrimetric assay, and the specific activities are reported in units/mg protein where one unit is 1 μmole fatty acid produced per minute.

TABLE 3
***Penicillium* sp. UZLM-A Lipase Activity Against Diglycerides**

Glyceride	Units/mg	% of the reactive triglyceride activity[a]
Triolein	778	100.0
Diolein (90% 1,3-DO)	160	20.6
Monoolein (100% 1-MO)	75	9.6
Tricaproin	1127	100.0
Dicaproin (*rac*)	62	5.5
Tripalmitin	25	100.0
1,3-Dipalmitin	7	28.0

[a]Lipase activity determined titrimetrically as described in the Materials and Methods section.

Substrate specificity. Comparison of initial hydrolysis rates of various lipid substrates shows that this enzyme strongly prefers tributyrin to any other lipid tested (Fig. 2). Most other substrates ranging from $C_{6:0}$ to $C_{18:2}$ are hydrolyzed at about 25% of the rate observed for tributyrin. One exception is trimyristin ($C_{14:0}$), whose initial rate of hydrolysis is more than twice that of most of the other substrates and two-thirds that of tributyrin. The hydrolysis of tricaprylin ($C_{8:0}$) rapidly stops about 15–30 s after the reaction is initiated, making measurement of the initial rate difficult. The reason for this behavior is uncertain. Although unlikely, the substrate may contain an impurity which inhibits the lipase. Another possibility is that the enzyme is rapidly inhibited by the products of the hydrolysis as discussed below.

Initial hydrolysis rates of diglyceride substrates are roughly one quarter or less of the rate of hydrolysis of the corresponding triglyceride (Table 3). HPLC analysis of reaction products also shows that hydrolysis of triolein leads to accummulation of diolein and monoolein, indicating slower hydrolysis of both diglyceride and monoglyceride (Fig. 3).

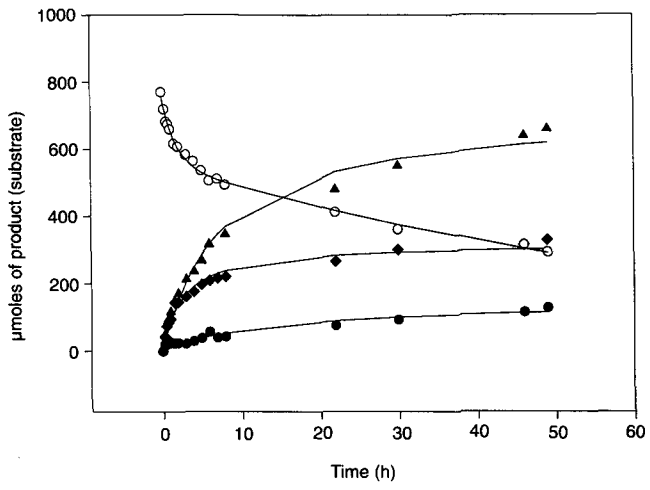


FIG. 3. Hydrolysis of triolein by *Penicillium* sp. UZLM-A lipase. The course of the reaction was followed by high-performance liquid chromatography analysis of a small aliquot of the reaction mixture at various time points. Triolein is represented by open circles, diamonds represent diolein, closed circles represent monoolein, and triangles represent oleic acid.

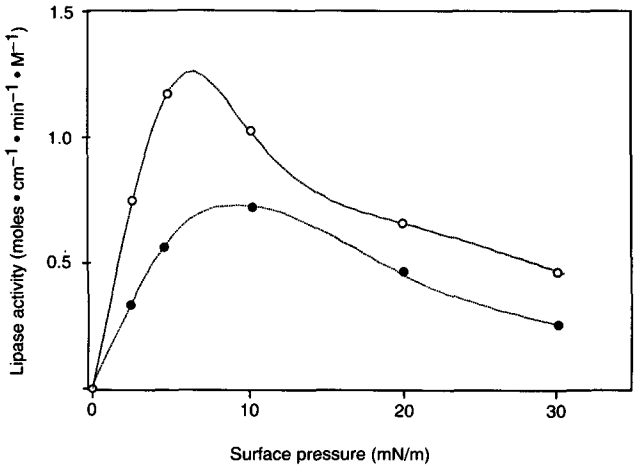


FIG. 4. Surface pressure profile of *Penicillium* sp. UZLM-A lipase determined by the monolayer method. Open circles represent curve for 1,2-dicaprin (S) as the substrate and closed circles represent 2,3-dicaprin (R) as the substrate.

1,2-Diolein is produced in about 5-fold excess over 1,3-diolein, indicating that this lipase shows a strong preference for hydrolysis of the acyl chains at the 1,3 positions.

The monolayer studies using dicaprin as a substrate show a rather unusual property of UZLM-A lipase (Fig. 4). Most lipases show their highest activities at surface pressures of 20–40 mN/m and lose their activity when exposed to the interface at a low surface pressure (5 mN/m) (37). In contrast, the *Penicillium* lipase prefers a low density monolayer film. This lipase also showed a weak S (1,2-dicaprin) over R (2,3-dicaprin) enantiomeric preference (Fig. 4) and strong 1,3-positional selectivity. Monolayer studies using other synthetic lipids as substrates confirmed the strong 1,3-positional selectivity and the weak S stereoselectivity. The enantiomeric selectivity was mainly observed at low interfacial surface pressure, similar to the properties of other lipases reported by Rogalska *et al.* (37).

The preference of UZLM-A for triglycerides and low interfacial pressures suggests an explanation for the rapid loss of activity against tricaprylin. The inhibition might result from accumulation of reaction products which increases the surface pressure at the interface. In the lipase assay used in this study, PDMS was used as a carrier for the triglycerides and the emulsions of the triglyceride–PDMS complex were used as the lipase substrate. In this type of emulsion, triglycerides and products of the lipolysis desorb only slowly from the surface of the emulsion droplet. The desorption rate is inversely proportional to the hydrocarbon chain length and is higher for fatty acids, mono- and diglycerides than for triglycerides. At least transiently, however, mono- and diglycerides could accummulate to create a high interfacial surface pressure. The surface pressures exerted by mono- and diglycerides are known to be higher than for monolayers of triglycerides and could inhibit UZLM-A, which shows a preference for low surface pressure interfaces. Perhaps the dicaprylin–PDMS complex forms the most impenetrable interface and accumulation of dicaprylin in

the course of the lipolysis produces a reaction profile typical of product inhibition.

DISCUSSION

A number of different lipases have been purified from various strains of *Penicillium*. Some of the properties of lipase UZLM-A are similar to other *Penicillium* lipases. Like UZLM-A, many *Penicillium* lipases have reported MW in the 25,000–32,000 range. The MW of 27,344 determined for the lipase UZLM-A is the most similar to the MW of 27,000 estimated from gel filtration of lipase A from *P. cyclopium* Westring (22). Interestingly, these two lipases also share similarities in their substrate preferences. In both cases, tributyrin is hydrolyzed faster than any other triglycerides tested. Lipases with preferences for tributyrin have also been described from *P. crustosum* (MW 32,000) (21) and from *Penicillium citrinum* (no MW estimate provided) (26). The lipase from *P. expansum* (MW 25,000) also reportedly hydrolyzes triglycerides faster than other substrates (28). The similarity of the *N*-terminal sequences of *P. expansum* lipase and UZLM-A strongly suggests that they are similar enzymes. No sequence information is available for the *P. cyclopium* and *P. citrinum* enzymes, but based on the similarities to UZLM-A and *P. expansum* lipase cited above, one might also also expect sequence similarities. Cloning and expression of the lipase described here are underway and should soon provide a complete amino acid sequence.

In contrast to the similarities to some *Penicillium* lipases, there are also striking differences between UZLM-A and other *Penicillium* lipases. One lipase from *P. cyclopium* M1 and one from *P. simplicissimum* are considerably larger than other *Penicillium* lipases, 54,000 and 56,000 MW, respectively (24,27). *Penicillium cyclopium* M1, *P. cyclopium* Westring, and *P. camembertii* produce enzymes which hydrolyze mono- and diglycerides and monoacyl esters significantly faster than they hydrolyze triglycerides (23,25), the opposite of the preferences described here. The *N*-terminal sequence of *P. camembertii* lipase bears no resemblance to that of UZLM-A and *P. expansum* lipase. Despite some similarity of the *N*-terminal sequence of our lipase to a region in the middle of *P. camembertii* lipase, there is probably no functional significance to the similarity, and they probably represent truly different lipases. *Penicillium cyclopium* M1 and *P. cyclopium* Westring produce both lipases that prefer triglycerides and lipases that prefer mono- and diglycerides, and complete hydrolysis of triglycerides to fatty acids and glycerol requires the presence of both lipases (23). A similar situation is likely to exist in many other *Penicillium* strains as well.

One of the most interesting features of lipase UZLM-A is its activity and stability at low surface pressures. This type of behavior could explain why this enzyme prefers liquid triglycerides (oils) over solid fats which exert a higher surface pressure. The affinity of UZLM-A lipase to low surface pressure interfaces could also explain its low activity against di- and monoolein. The surface pressures exerted by both diglycerides and monoglycerides are 2–3 times higher than the maximal surface pressure of the triglyceride (38). The lipase from *P. camembertii*, in contrast to UZLM-A, maintains its activity at surface pressures above 20 mN/m, consistent with its high activity on mono-and diglycerides (39). The surface properties and stereoselectivities of other *Penicillium* lipases have not been reported and possibly have not been extensively investigated. The three-dimensional structure of *P. camembertii* lipase has been reported (11). Crystal structure analysis of UZLM-A is currently underway. Comparison of these structures should provide important insight into the substrate selectivities of lipases.

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Sphingomyelin Metabolism Is Linked to Salt Transport in the Gills of Euryhaline Fish

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ABSTRACT: By *in vivo* and *in vitro* studies of L-(3-^{3}H)serine and [9,10(n)-^{3}H]palmitic acid incorporation into phospholipids, we show a change in the renewal of the ceramide moiety of sphingomyelin in the gills of euryhaline fish (sea bass and eels) when the animals were subjected to abrupt alterations in environmental salinity. *In vivo*, decrease of the salinity from sea water (salinity 3.7%) to diluted sea water (salinity 1%) induced an increase of label incorporation into gill sphingomyelin. The same was true when gills from sea water-adapted sea bass or sea water-adapted eels were incubated in diluted sea water. A decrease in free ceramides synthesis was also observed in the gills of sea water-adapted sea bass when the salinity of the incubation medium was reduced. Direct inhibition of Na^+/K^+-ATPase activity with ouabain decreased the sphingomyelin synthesis in the gills of sea bass during *in vitro* incubation in diluted sea water, whereas treatment with furosemide stimulated sphingomyelin synthesis in the same gills incubated in sea water. These findings indicate that changes in Na^+ fluxes modify the sphingomyelin turnover and control the production of free ceramides and sphingosine in gill cells of euryhaline fish. In view of the well-known effects of these sphingomyelin degradation products on isolated tumor cell differentiation, we suggest that they play a very important role in modulating chloride cell distribution and metabolism of fish gills during abrupt changes in environmental salinity.

Lipids 31, 385–392 (1996).

Sphingomyelin (SPH) is found in all fish and crab tissues as a component of cell membranes (1–4). There is a greater percentage of SPH in gill phospholipids than in those of other tissues (2,4), and sea water (SW) adaptation increases this percentage in the gills of euryhaline crabs (5). The biosynthetic pathway for SPH appears to be very active in the gills of teleosts and crustaceae (6,7). This pathway involves ceramide synthesis from long-chain bases followed by transfer of a phosphocholine moiety catalyzed by phosphatidylcholine (PC)-ceramide cholinephosphotransferase (8). Factors influencing SPH turnover include serine and acyl-CoA availability, and the relative activities of key enzymes in the synthesis and degradation pathways. The first step in degradation involves the removal of the head-group by sphingomyelinase, an enzyme found in gill cells (2).

In addition to their respiratory function, the gills of euryhaline fish and crabs participate in osmoregulation (9,10). The gills are the site of active NaCl transport and the direction of active salt transport is correlated with external salinity. In gills of *Anguilla anguilla* and *Platichthys flesus*, two euryhaline teleosts, active NaCl absorption begins during adaptation to fresh water (FW) (11,12). The branchial epithelium of SW-adapted fish show a much larger population of chloride cells than that of FW-adapted fish (13). These cells are the site of chloride transport and possess enzymatic characteristics of electrolyte-transporting epithelia (13,14). In gill epithelium, most of the Na^+/K^+-ATPase activity is found in chloride cells, and cell density depends directly on the external salinity (13,14). In both teleosts and crabs, changes in chloride cell density are accompanied by an alteration of phospholipid metabolism (5,15). It has been reported (8) that SPH concentration is different in gills of FW- and SW-adapted crabs (*Eriocheir sinensis*). Changes in the concentration of this major phospholipid may reflect modification of its turnover rate. Moreover, SPH catabolism liberates ceramide and/or free sphingosine, potential second messengers which could modulate cell function (16,17). The recent discovery that these intermediates play an important role in controlling cell growth, differentiation and oncogenesis (17–20) has motivated this study of SPH metabolism during abrupt salinity changes in fish gills.

MATERIALS AND METHODS

Two different species of euryhaline fish were used in this study, a marine fish: sea bass (*Dicentrarchus labrax*) and a freshwater fish: yellow European eel (*A. anguilla*). Sea bass (*D. labrax*) averaging 75 g were originally obtained from a sea farm (Centre de production aquacole IFREMER, Palavas les Flots, France) and were kept in indoor tanks with continuously flowing Mediterranean SW (salinity 3.7%) at 14–15°C for 4 wk. Eels averaging 100 g were originally obtained from the Rhône river and were acclimated to continuously flowing

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Abbreviations : Amiloride, N-amidino-3,5-diamino-6-chloropyrazinecarboxamide; furosemide, 5-[aminosulfonyl]-4-chloro-2-[(2-furanylmethyl)-amino] benzoic acid; FW, fresh water; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SPH, sphingomyelin; SW, sea water; TLC, thin-layer chromatography; TPA, 12-0-tetradecanoylphorbol 13-acetate.

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FW or SW at 14–15°C for 4 wk. Sea bass were fed a standard diet and eels were starved.

In vivo *L-3(^{3}H)-serine incorporation.* Sea bass acclimated to SW received 100 μCi of L-3(^{3}H)-serine (29 Ci /mmol; Amersham, Les Ulis, France) per 30 g wet weight by intraperitoneal injection and were randomly divided in two groups: (i) SW (salinity 3.7%) and (ii) diluted SW (salinity 1%) at a controlled temperature of 17°C. After 16, 24, and 36 h, the animals were killed by a blow on the head, and gills were isolated and weighed. Lipid extraction and purification were performed on the fresh tissue by the method of Folch *et al.* (22), modified by Chapelle *et al.* (23). The phospholipid classes were resolved by two-dimensional thin-layer chromatography (TLC) on precoated silica-gel G-60 plates (Merck, Darmstadt, Germany) as previously described by Portoukalian *et al.* (24). Phospholipids were detected with a specific spray reagent (25) and quantified by determining the phosphorus content in each spot as previously described (5,24). For the radioactive determination, phospholipid spots revealed on the TLC chromatograms were counted in the presence of silica gel, by scraping into a scintillation vial and resuspending in 3 mL water/ethanol (1:1, vol/vol) and 8 mL Picofluor 30 (Packard, Downers Grove, IL) (26).

In vitro *L-3(^{3}H)-serine incorporation.* Gills from SW-adapted sea bass were cut at their bases, and each pair transferred to ultra-filtered Mediterranean SW or diluted SW, rinsed and blotted on paper. Then, one gill of each pair was placed in SW and the other in diluted SW containing 0.3 μM L-3(^{3}H)-serine (29 Ci/mmol; Amersham) or 0.2 μM [9,10(n)-^{3}H]-palmitic acid (40–60 Ci/mmol; Amersham) and incubated at 15°C for the indicated time. Lipids were extracted and analyzed as described above.

Analysis of long-chain bases in SPH. Phospholipids were separated by one dimensional TLC on precoated silica-gel G-60 plates in chloroform/methanol/water (65:35:6, by vol). The SPH spots, visualized with iodine, were scraped into tubes, eluted with a mixture of chloroform/methanol/acetic acid/water (50:39:1:10, by vol) and washed as described by Arvidson (27). Some lipid extracts were subjected to mild alkaline methanolysis (28) before purification of SPH by TLC. SPH was first enzymatically hydrolyzed using 10 units/mL phospholipase C (Type 1 from *Clostridium perfringens*; Sigma, St. Louis, MO) in 1 mL of Tris pH 7.4, 30 mM $CaCl_2$ mixed with 1 mL diisopropyl ether for 18 h at 20–25°C to obtain the ceramides (29). Diisopropyl ether was evaporated in a stream of nitrogen before the addition of 4 mL chloroform/methanol (1:1, vol/vol) and 1.8 mL water. Ceramides were extracted in the chloroform phase, followed by centrifugation. Aliquots of the water and chloroform soluble extracts were counted. Under these conditions of hydrolysis, TLC of the chloroform extract on silica gel in chloroform/methanol/water (65:35:6, by vol) showed the total disappearance of SPH. The ceramides were acid hydrolyzed in teflon-lined screw-capped tubes in concentrated HCl/water/methanol (1:2:6, by vol) for 18 h at 80°C (30). The samples were taken to dryness with added methanol and benzene to eliminate HCl and water. The residues, redissolved in 0.5 mL of chloroform/methanol (2:1, vol/vol) and diluted with standards (mixture of SPH, ceramides, sphingosine, fatty acids, and esters), were separated by one-dimensional TLC on precoated 10 × 5 cm TLC plates. The plates were first developed to 5 cm in chloroform/methanol/water (100:42:6, by vol) and then, after drying, in chloroform/methanol (50:3, vol/vol) to the 10-cm length. The fatty acids and esters, ceramides, sphingosine, and SPH spots were visualized with iodine and scraped into scintillation-counting vials and counted. Under these conditions, all of the radioactivity initially found in the ceramide was distributed between sphingosine and fatty acids, according to the radioactive precursor.

Determination of radioactivity in free ceramides of gills. For free ceramide measurements, lipids were subjected to mild alkaline hydrolysis (28) in 0.4 M methanolic NaOH at 37°C for 1 h. Concentrated HCl was added to obtain a final concentration of 0.4 N acid, and hydrolysis was continued for 1 h at the same temperature. After extraction with chloroform, the resulting extracts were diluted with solutions of purified ceramides and separated by one-dimensional TLC on 10 × 5 cm precoated silica-gel plates by two successive developments, as described above. SPH and ceramides were visualized with iodine, scraped, and counted. The ceramide radioactivity in tissue, in dpm/100 mg wet gills, was calculated from SPH radioactivity found on the plate.

Statistics. Means for individual phospholipid radioactivity were compared using one-way analysis of variance. Percentages were arcsine transformed. If test for normality failed, then a nonparametric Kruskal-Wallis one-way analysis of variance on ranks was used. All values presented are means ± SEM.

RESULTS

In vivo incorporation of L-(^{3}H)serine into gill lipids of the sea bass during abrupt change in salinity from SW (3.7%) to diluted SW (1.0%) is summarized in Table 1. During this 36-h experimental period, 90% of lipid radioactivity in the gills was present in phospholipids, and this stayed relatively constant [from 7318 ± 293 (4) dpm/100 mg wet gills at 16 h to 8031 ± 250 (4) at 36 h in SW and 6143 ± 288 (4) to 7655 ± 774 (4) in diluted SW]. When the distribution of the radiolabel among lipid species was determined (see Table 1), radioactivity was significantly lower ($P < 0.01$) in SPH of the gills of fish in SW than in those of fish transferred to diluted SW. No major variations for the other lipids were observed. The gross distribution of radioactivity in PC, phosphatidylserine (PS), and phosphatidylethanolamine (PE) appeared to be independent of salinity. In these same experiments, phospholipid composition showed the same general pattern in the gills of both animals, with SPH representing 11.5 ± 0.2% of total lipid phosphorus [PC: 46.0 ± 0.3%, PS: 6.4 ± 0.2%, phosphatidylinositol (PI): 4.4 ± 0.2%, and PE: 17.2 ± 0.5%]. To determine whether the differences in SPH metabolism during the abrupt decrease in salinity resulted from a direct effect on

TABLE 1
***In vivo* Distribution of L(3-^{3}H)Serine into Gill Lipids of the Sea Bass During an Abrupt Change from Sea Water (SW, salinity 3.7%) to Diluted Sea Water (salinity 1%)[a]**

Lipid	SW	Diluted SW
SPH	9.00 ± 0.17[b]	11.12 ± 0.10[c]
PC	18.16 ± 0.57	18.51 ± 0.42
PS	38.54 ± 0.81	37.74 ± 0.55
PE	14.64 ± 0.28	15.78 ± 0.33
NL	12.53 ± 0.50	10.71 ± 0.44

[a]Twenty-four fish adapted to SW for at least 6 wk received 100 µCi each of L-(3-^{3})serine by intraperitoneal injection and were kept in aerated SW or diluted SW tanks at a controlled temperature of 17°C; four were killed 16, 24, and 36 h after the initial injection. The gill lipids were extracted and analyzed as described in the Materials and Methods section.
[b]Values are expressed as dpm% of the total lipid extracted from the gills and are the means ± SEM of 12 animals.
[c]Values statistically different, $P < 0.01$, between SW and diluted SW; SPH, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; NL, neutral lipids.

gill SPH turnover, we have carried out *in vitro* experiments, looking at the effect of medium salinity on the incorporation of L-(^{3}H)serine into phospholipids of isolated gills from SW-adapted fish. As shown in Table 2, there was a marked increase (+43%) in SPH radioactivity (from 11.6 to 16.4%) in phospholipids extracted from gills incubated in diluted SW. Low salinity resulted in a decrease (−13%) in PS radioactivity without modifying PC and PE label percentage.

To confirm the effect of abrupt change in external salinity on gill SPH metabolism in another euryhaline fish, we have analyzed the *in vitro* incorporation of L-(^{3}H)serine into the phospholipids of gills obtained from SW- and FW-adapted eels. In these experiments, gills were incubated in a synthetic medium with an osmotic pressure and ionic composition similar to eel plasma. There was a large difference between the incorporation of the radiolabel into SPH of gills from SW- or FW-adapted eels (Fig. 1). There were no major differences between the other phospholipids, except for a small increase of the label in PC of gills from SW-adapted eels.

The distribution of the label in SPH isolated from gills after *in vivo* radioactive serine incorporation revealed that

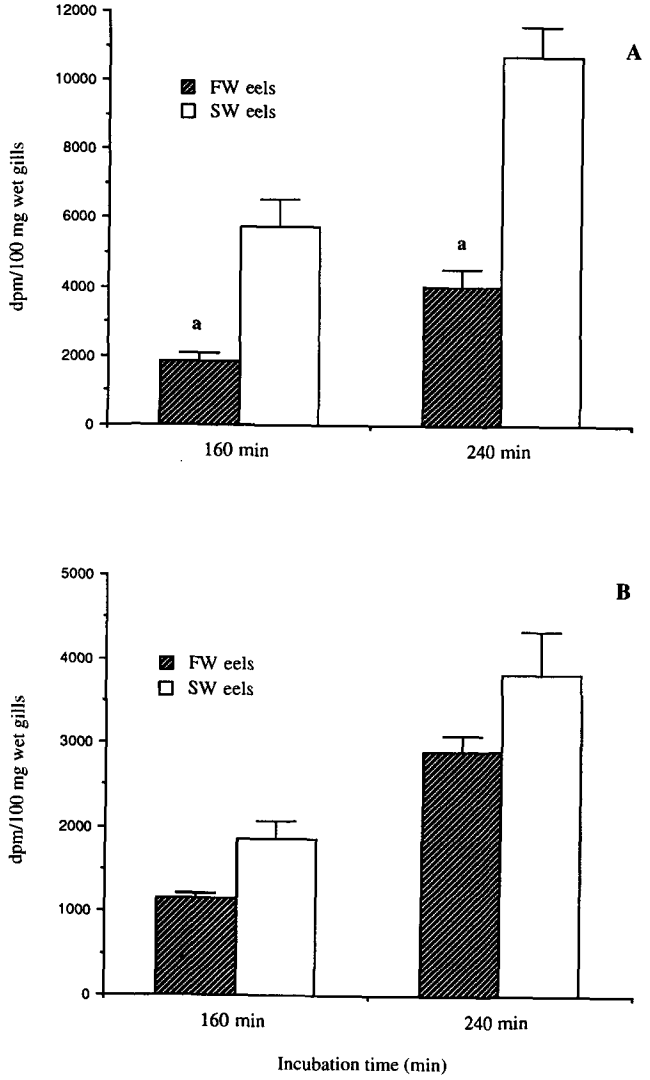


FIG. 1. *In vitro* incorporation of L-(U^{14}C)serine into gill sphingomyelin (A) and phosphatidylcholine (B) of salt water (SW)- and fresh water (FW)-adapted eels. Gills from SW- or FW-adapted eels were incubated for 160 and 240 min at 17°C in a synthetic medium of NaCl, 147 mM; KCl, 2.66 mM; $CaCl_2$, 2.17 mM; $MgCl_2$, 2.34 mM; HNa_2PO_4, 8.1 mM; and H_2NaPO_4, 1.9 mM, containing 0.35 µCi L-(U^{14}C)serine/mL. Values are means ± SEM of four animals. a, Values statistically different ($P < 0.01$) between gills from SW- and FW adapted eels.

TABLE 2
Influence of the Salinity of *in vitro* Incubation Medium on the Distribution of L-(3-^{3}H)Serine into Gill Phospholipids of Sea Bass[a]

	Incubation medium	
Lipid	SW	Diluted SW
SPH	11.56 ± 0.19[b]	16.40 ± 0.06[c]
PC	4.48 ± 0.27	5.20 ± 0.06
PS	48.03 ± 1.32	41.58 ± 0.79[d]
PE	9.87 ± 0.76	10.79 ± 0.35

[a]Gills from SW-adapted sea bass were incubated for 4 h at 17°C in SW (salinity 3.7%) or diluted SW (salinity 1%) containing 5 µCi L-(3-^{3}H)serine/mL.
[b]Values are expressed as dpm% of the total dpm in the lipid extracted from the gills and are the means ± SEM of six animals. Values statistically different: [c]$P < 0.01$, [d]$P < 0.05$, between SW and diluted SW. See Table 1 for abbreviations.

about 80–85% of total radioactivity was found in the sphingosine moiety. The fatty acids and choline isolated during enzymatic and acid treatments contained 10–15% of the radioactivity. After *in vitro* incubation, 90–95% of the radioactive label was found in the sphingosine moiety of the purified SPH. These results were consistent with the sphingosine biosynthetic pathway, in which the first stage is a condensation of serine and palmitoyl-CoA followed by an acylation to ceramide. To form SPH, the phosphorylcholine head-group seems to be transferred from PC to ceramide (6). To determine if the rate of the acylation step in the SPH biosynthesis was altered by the salinity change, we have measured the incorporation of (^{3}H)palmitic acid into gill SPH under the same

in vitro conditions. Results of these experiments with gills from sea bass are summarized in Figure 2. The salinity of the incubation medium had a large effect on the rate of radioactive palmitic acid incorporation into gill SPH. For the other lipids, no differences between SW and diluted SW were observed except after 2 h of incubation in PC. In these experiments, 27% of the radiolabel was located in sphingosine and 73% in the acyl moiety of the SPH isolated after SW or diluted SW incubation.

The above results demonstrate an effect of external salinity on the rate of SPH synthesis in gills of sea bass and eels. However, they give only an imperfect picture of the biochemical changes due to salinity variations. For this reason, it was

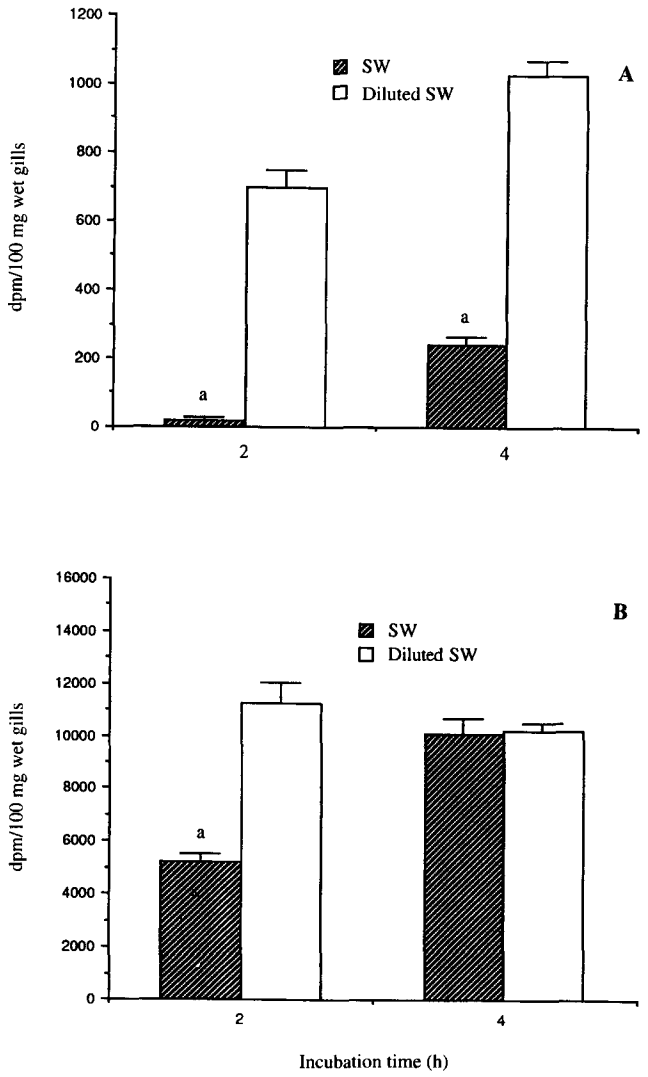


FIG. 2. Influence of medium salinity on the incorporation of [9,10(n)-^{3}H]palmitic acid into (A) gill sphingomyelin and (B) phosphatidylcholine of sea bass. Gills from SW-adapted sea bass were incubated for 2 and 4 h at 17°C in a SW or diluted SW medium containing 5 μCi (^{3}H)palmitic acid/mL. Values are means ± SEM of four animals. a, Values statistically different ($P < 0.01$) between gills from SW (salinity 3.7%) and diluted SW (salinity 1%) incubation medium. See Figure 1 for abbreviation.

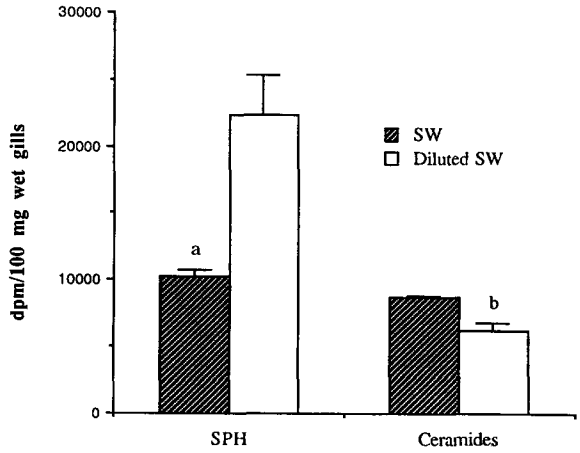


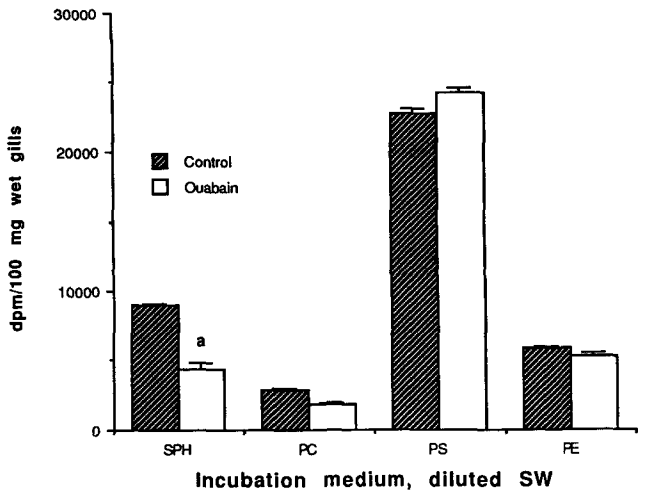
FIG. 3. Sphingomyelin and ceramide radioactivities in gills from SW-adapted sea bass after 4 h of incubation at 17°C in SW or diluted SW containing 15 μCi (^{3}H)serine/mL. Values are means ± SEM of four animals. Values statistically different: a, $P < 0.01$ and b, $P < 0.05$, between gills from SW and diluted SW incubation media; SPH, sphingomyelin. See Figure 1 for other abbreviation.

of interest to measure radioactivity incorporated into free ceramide, an intermediate of SPH metabolism. Figure 3 shows the activity of SPH and ceramides in the gills of sea bass after 4 h of incubation in diluted SW or SW containing 15 μCi (^{3}H)serine/mL. We observed an effect of external salinity on the incorporation of radiolabel into free ceramides and SPH. Diluted SW caused a decrease in label incorporation into tissue free ceramides (essentially with nonhydroxylated fatty acids) together with an increase in incorporation into SPH.

To test the hypothesis that the increase in SPH biosynthesis is linked with the change of Na^+ flux in branchial cells during salinity changes, we have studied the effect of Na^+ transport modulators on *in vitro* incorporation of L-(^{3}H)serine into SPH of sea bass gills. The effect of ouabain on L-(^{3}H)serine incorporation into gill phospholipids of sea bass incubated in diluted SW is summarized in Figure 4. Ouabain (1 mM) decreased the incorporation of the label into gill SPH (from 16.4 ± 0.5 to 11.9 ± 0.9%) after 4 h of incubation. In the same medium, neither 1 mM N-amidino-3,5-diamino-6-chloropyrazinecaarboxamide (amiloride), nor 1 mM 5-[aminosulfonyl]-4-chloro-2-[2-furanylmethyl)-amino] benzoic acid (furosemide) affected the SPH synthesis. Under the same conditions, however, furosemide strongly increased the incorporation of the label into PC (see Fig. 5). During incubation in SW, only furosemide increased the incorporation of the radiolabel into SPH from 7.6 ± 0.3 to 10.6 ± 0.5%; the other Na^+ modulators had no effect (Fig. 5).

DISCUSSION

This study shows that an abrupt decrease in salinity causes a marked increase in SPH turnover in the gills of euryhaline fish. However, this effect is probably transient because maximal changes appear to occur within 6–48 h. The same pattern



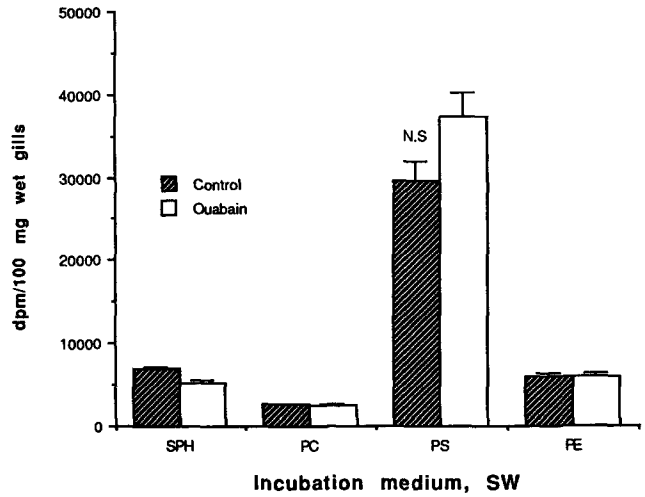
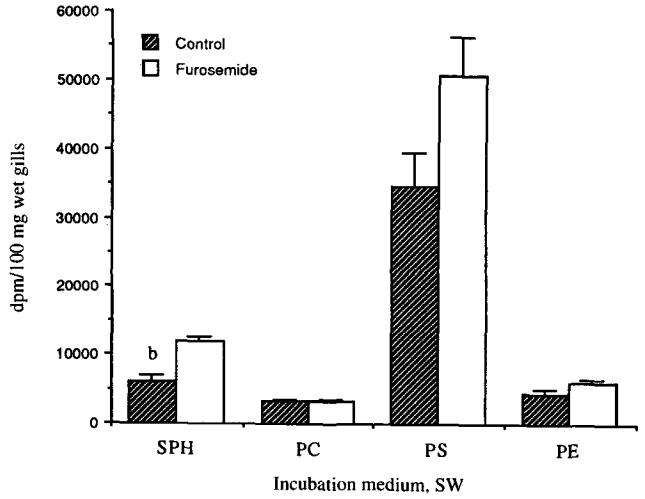


FIG. 4. Effect of 1 mM ouabain on L-(3-^{3}H)serine incorporation into gill phospholipids of sea bass during a 4-h *in vitro* incubation in diluted SW. Values are means ± SEM of four animals. a, Values statistically different ($P < 0.01$) between control and ouabain-treated gills; N.S., not significant. See Figure 1 for other abbreviation.



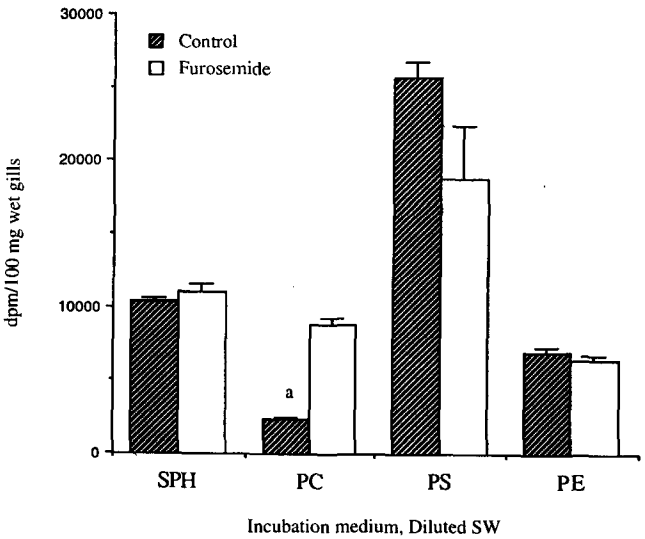


FIG. 5. Effect of 1 mM 5-[aminosulfonyl]-4-chloro-2-[(2-furanylmethyl)-amino] benzoic acid (furosemide) on L-(3-^{3}H)serine incorporation into gill phospholipids of sea bass during a 4-h *in vitro* incubation in SW or diluted SW. Values are means ± SEM of four animals. Values statistically different: a, $P < 0.01$ and b, $P < 0.05$, between control and furosemide-treated gills; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine. See Figures 1 and 3 for other abbreviations.

is observed in SW- or FW-adapted eels (2). However, 16–36 h after the transfer of SW-adapted sea bass to diluted SW, the percentage of radioactivity incorporated into SPH increases by 24%. Four hours after the salinity change *in vitro*, radioactivity incorporation increases by 110% for SPH and decreases by 36% for free ceramide, an intermediate metabolite. These results demonstrate that an alteration in ceramide metabolism induced by an abrupt change of salinity takes place in the gills of euryhaline fish and that it may mediate some of the cellular rearrangement known to occur after an osmotic shock. Indeed, the number of chloride cells in the gills of euryhaline fish transferred from FW to SW and of hyperosmotic crab transferred from SW to FW increases (13,31).

Relatively little is known about the control of proliferation and distribution of the mitochondria-rich chloride-transporting cells in gills. The induction of the differentiation program of these cells is started by an unknown mechanism. In murine erythroleukemia cells, Kiss *et al.* (32) have shown that treatment with phorbol esters at concentrations sufficient to induce cell differentiation significantly increased the rate of SPH synthesis. In the same cells, the drop of intracellular Na^+ induced by dimethylsulfoxide appears as an early event of cell differentiation (33), and the decrease in Na^+/K^+ pump activity measured by Mayer and Bernstein (34) is probably one of the multiple factors inducing the differentiation program. Similarly, amiloride (a specific inhibitor of Na^+/H^+ exchange) interferes with 12-0-tetradecanoylphorbol 13-acetate (TPA)-induced differentiation of HL-60 cells (35).

We have shown that SPH biosynthesis *in vitro* by gills from SW-adapted sea bass, can be rapidly elevated by incubation in diluted SW. A similar effect has been noted by Girard *et al.* (36) for chloride cells from the gills of SW- compared with FW-adapted eels, in a study of the effect of adrenalin on ^{32}P incorporation into phosphatidic acid and PI.

Under our experimental conditions, the use of different inhibitors of Na^+ transport allowed additional observations. In FW or diluted SW, the pump transporting K^+ from the extracellular space into the cell is ATP dependent and Na^+ coupled (37). This Na^+/K^+ pump is inhibited by ouabain which acts on the basolateral membrane of the transporting cells (38,39). This ouabain-inhibited Na^+/K^+-ATPase is present in the gills of fish, and its activity is strongly enhanced by adaptation to SW in euryhaline fish (40). On the other hand, in FW, the active Na^+ uptake in trout gills is also inhibited by amiloride or furosemide, a second group of Na^+ transport modulators (41). These diuretics inhibit the active transfer of Na^+ on the apical membrane of the ion-transporting cells (42). With SW-adapted sea bass, 1 mM amiloride has no effect on the activation of SPH synthesis observed during *in vitro* incubation in diluted SW medium. It is interesting to note that amiloride has practically no effect on the cellular sodium movements under these conditions (42). Concentration of the Na/K pump inhibitor ouabain, which causes a reduction in sodium efflux (38), has an inhibitory effect on the radiolabel incorporation. Furosemide, another loop diuretic, activates the SPH synthesis in SW but has no effect in diluted SW.

The transient increase in SPH synthesis observed in the gills of euryhaline animals during a sudden decrease of salinity probably corresponds to the increased turnover of a second messenger provided by SPH metabolism in some specialized cells of the gills. This is the first time that the modulation of SPH metabolism in a specialized organ has been linked directly with environmental changes.

Lavie *et al.* (43) have shown in various cultured cell types that the replacement of cell-conditioned medium by HEPES-buffered saline causes a rapidly increase in the levels of endogenous sphingoid bases. In both isolated fish gills and cultured cells, activation or inhibition of the SPH cycle may also be triggered pharmacologically or by changing medium conditions. At least three components of this cycle act as biological signals in cells: ceramides, sphingosine, and sphingosine-1-phosphate (44). The biochemical targets of these intermediates in synthesis or degradation pathways of SPH are unknown; however, ceramides and sphingoid bases act as modulators of protein kinase C activity (45), and these compounds have been shown to regulate cell growth, cell differentiation, programmed cell death and eicosanoid production (17,18).

SPH turnover is influenced by the availability of precursors and by the relative activities of key enzymes in the synthesis and degradation pathway. The synthesis is dependent on the activity of sphingomyelin synthase which transfers the phosphorylcholine head group from PC to ceramide, yielding SPH and diacylglycerol (6,46). The degradation pathway is influenced by the activity of pH-sensitive sphingomyelinase, an enzyme found in plasma membranes and in lysosomes (47), which catalyzes the removal of the SPH head-group to give ceramides. In view of our results, we can hypothesize that the activity of these enzymes in the gills of euryhaline fish may be modulated by changes in intracellular Na^+ or pH induced by variation in external salinity. If this is the case, the increase of SPH turnover (caused by the activation of SPH synthase and/or sphingomyelinase during transfer to diluted SW) may control the levels of intermediate compounds such as sphingoid bases, ceramides or diacylglycerol. In turn, these intermediates would act as signals controlling protein kinase C activity, thereby initiating the activation or inhibition of chloride cell differentiation. The report by Kolesnick (48), in which sphingomyelinase prevents the morphologic changes associated with TPA-induced conversion to the macrophage phenotype in HL-60 cells, supports this hypothesis. However, in HL-60 sensitive-cells, Okazaki *et al.* (49) found that 1-α,25-dihydroxyvitamin D_3 activates a sphingomyelinase that increases SPH turnover and causes the formation of intracellular ceramides, which then induce cell differentiation to the monocytic phenotype. These observations do not allow identification of the intermediate of the SPH pathway, which controls chloride cell proliferation in the gills of euryhaline fish during abrupt changes in salinity.

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Studies on Phospholipid Biosynthesis in Hepatocytes from Alcoholic Rats by Using Radiolabeled Exogenous Precursors

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ABSTRACT: We have studied the synthesis of phospholipids in hepatocytes isolated from chronically ethanol-treated rats by using isotopically labelled serine, ethanolamine, and choline as exogenous precursors. Our results demonstrate that ethanol induces specific effects on the biosynthesis of phosphatidylethanolamine and phosphatidylcholine *via* CDP-derivatives and also on the synthesis of phosphatidylserine *via* the Ca^{++}-dependent base-exchange reaction. Thus, the synthesis of phosphatidylethanolamine from [^{3}-H]ethanolamine and the incorporation of [^{3}H]serine into phosphatidylserine were clearly higher in hepatocytes from ethanol-treated rats compared to controls. The synthesis of phosphatidylcholine from [methyl-^{14}C]choline, on the other hand, decreased markedly, suggesting a specific inhibition of cholinephosphotransferase activity. We have also demonstrated that the phosphatidylcholine levels are markedly decreased in hepatocytes isolated from chronically ethanol-treated rats as a consequence of the lower phosphatidylcholine biosynthesis. The decrease in the incorporation of radioactivity from choline to betaine, which we also found, is interpreted as being the result of a higher use of betaine as methyl donor instead of methionine to maintain the hepatic *S*-adenosylmethionine levels in chronic alcoholism.
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Phospholipids are basic components of cell membranes, acting mainly as regulators of membrane-bound enzymes and in the membrane transport processes. The pathways of glycerophospholipid biosynthesis in the mammal liver are well established (1), and their regulation is becoming understood in considerable detail (1,2).

It is also widely recognized that chronic ethanol administration produces two major disturbances in lipid metabolism, fatty liver, and hyperlipidemia. Fatty liver develops as a consequence of alterations in the regulation of several metabolic pathways in the liver while hyperlipidemia can develop from alterations in metabolic pathways in hepatic and extrahepatic tissues (reviewed in Refs. 3 and 4). As far as fatty liver is concerned, it has been consistently demonstrated that ethanol induces triacylglycerol synthesis and its accumulation in the liver after both acute and chronic treatment (5). Fatty acids accumulate because of various different alterations and from several sources: enhanced hepatic lipogenesis, decreased lipoprotein release, increased mobilization of peripheral fat, and decreased fatty-acid oxidation as a consequence of reduced citric-acid-cycle activity (for review see Ref. 6).

As mentioned above, hepatic triacylglycerol accumulation has been consistently described in chronic alcoholism; however reports on the effects of ethanol on the biosynthesis of phospholipids are scarce and contradictory. Castro *et al.* (7) reported that acute ethanol administration stimulates the synthesis of phospholipids in rat hepatocytes, while other authors have indicated a marked decrease in the biosynthesis of phosphatidylcholine in alcoholic rat liver (8). This reduction in phosphatidylcholine synthesis is consistent with the decrease found by Lieber *et al.* (9) in the hepatic phosphatidylcholine levels after chronic ethanol treatment. In spite of the physiological importance of phospholipids, the mechanism by which ethanol interferes with their metabolic pathways has not been adequately studied. Thus, we have made a comparative study into the effects of ethanol on the specific biosynthetic pathways of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine by using radiolabelled serine, ethanolamine, and choline as exogenous precursors.

EXPERIMENTAL PROCEDURES

L-[3-^{3}H]serine, [1-^{3}H]ethanolamine, and [methyl-^{14}C]choline were purchased from Amersham International (Amersham, Bucks, United Kingdom). Fatty acid-free bovine serum albumin (BSA) and collagenase (Type A from *Clostridium histolyticum*) were supplied by Boehringer Mannheim (Barcelona, Spain). Thin-layer chromatography (TLC) plates were from Sigma (Madrid, Spain) and Percoll was supplied by Pharmacia (Uppsala, Sweden). All the other reagents used were of analytical grade.

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Abbreviations: BSA, bovine serum albumin; TLC, thin-layer chromatography.

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Male Sprague-Dawley rats (150–170 g initial body weight) were fed for 31 d on the Miller *et al.* (10) liquid diet. In the ethanol-fed liquid diet, the caloric contribution of proteins, fats, carbohydrates, and ethanol was 23, 12, 29, and 36%, respectively. A pair-fed group received an isocaloric mixture with maltose–dextrin instead of ethanol.

The rats were housed in a controlled temperature and humidity chamber with a 12 h light/dark cycle. The rats were starved overnight when the hepatocytes were to be isolated.

The hepatocytes were isolated by Seglen's collagenase perfusion procedure (11) with minor modifications. Isolated hepatocytes were suspended in Krebs-Henseleit buffer, pH 7.4, supplemented with 1.5% BSA and 10 mM glucose. Their viability, determined by Trypan blue exclusion, was always higher than 90% in all the preparations used.

The hepatocytes were incubated at 37°C in a shaken water bath (80 oscillations/min) in Krebs-Henseleit buffer, pH 7.4, containing 1.5% BSA and 10 mM glucose, under an atmosphere of carbogen (95% O_2:5% CO_2). The incubations contained 5×10^6 cells in a final volume of 2.5 mL of incubation medium. The reactions were initiated by the addition of 125 nmol of methionine and L[3-^{3}H]serine (125 nmol; 150960 dpm/nmol) or [1-^{3}H]ethanolamine (100 nmol; 119920 dpm/nmol) or [methyl-^{14}C]choline (125 nmol; 32667 dpm/nmol). The incubations were carried out for 30 min at 37°C under an atmosphere of carbogen and stopped by the addition of 7.5 mL of ice-cold Krebs-Henseleit. The cells were washed twice in Krebs-Henseleit medium at $50 \times g$ for 5 min, and the pellet was collected to be used for the analysis of lipid and water-soluble metabolites.

Lipids were extracted from the cell pellet according to the procedure of Folch *et al.* (12). The chloroform layer was dried by evaporation under a stream of nitrogen, while the water–methanol phase was collected and used to determine water-soluble metabolites. The lipid residue was dissolved in chloroform, and the different phospholipids were separated on silica gel 60 G TLC plates, as indicated by Higgins (13). Silica gel bands corresponding to the individual phospholipids were scraped and used for phosphorous determination. Silica gel does not interfere with the colorimetric assay and the recovery by this procedure was higher to 90%. The levels of individual phospholipids were quantified by Bartlett's method (14) in control and ethanol-treated hepatocytes before being incubated in the presence of the different radioactive substrates.

Phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N,N*-dimethylethanolamine were isolated from the chloroform fractions by TLC on silica gel H plates, using chloroform/methanol/acetic acid (70:30:4, by vol) as solvent, according to Marino *et al.* (15).

The water-soluble intermediates, ethanolamine phosphate and CDP-ethanolamine, were separated from the aqueous fractions by Wang and Moore's method (16), using silica gel G TLC plates and a solvent system composed of 96% ethanol/3% NH_4OH (1:2, vol/vol). Betaine and the water-soluble phosphatidylcholine precursors, choline phosphate and CDP-choline, were separated from the aqueous fractions according to Vance *et al.* (17) using silica gel G TLC plates and methanol/0.6% ClNa/28% NH_4OH (50:50:5, by vol) as solvent.

The spots corresponding to each compound were visualized by exposing the plates to iodine vapors, then scraped and transferred to scintillation vials to measure the radioactivity with a Beckman 6000-TA liquid scintillation counter (Madrid, Spain).

The results are expressed as means ± SEM, as indicated in the legends to the tables. Statistical significance was determined by Student's *t*-test.

RESULTS AND DISCUSSION

We have analyzed the influence of chronic ethanol ingestion on the biosynthetic pathways of phospholipids in hepatocytes isolated from rat liver. The synthesis of phospholipids takes place *via* specific pathways; phosphatidylcholine and phosphatidylethanolamine can be synthesized in microsomes *via* CDP-derivatives while serine is incorporated into the headgroup of phosphatidylserine in a microsomal Ca^{++}-dependent base-exchange reaction. The phosphatidylserine is further decarboxylated in the mitochondria to give phosphatidylethanolamine *via* phosphatidylserine decarboxylase, and this phosphatidylethanolamine can be methylated to phosphatidylcholine by three enzymatic steps, which take place in the endoplasmic reticulum.

Firstly, to determine the effects of ethanol treatment on the levels of the individual phospholipids, they were isolated and quantified. The results in Table 1 demonstrate that the treatment produces a significant decrease in the amounts of phosphatidylcholine while the levels of the other phospholipids are similar in the control and ethanol-treated groups. These results are in accordance with those previously reported by other authors in both human (9) and primate (18) livers, suggesting a specific interference of ethanol on the phosphatidylcholine metabolism in different animal species.

It has been accepted that changes in the endogenous pools of intermediates may in some situations be responsible for the differences found in experiments in which the radiolabel incorporation is used as an index of biosynthetic processes. However, recent evidence has demonstrated the existence of different intracellular nonuniformly mixing metabolic inter-

TABLE 1
Effect of Chronic Ethanol Consumption on the Phospholipid Levels in Isolated Rat-Hepatocytes[a]

	Control	Ethanol
Phosphatidylcholine	3.05 ± 0.13	2.04 ± 0.16[a]
Phosphatidylethanolamine	2.14 ± 0.16	1.78 ± 0.17
Phosphatidylserine	0.84 ± 0.07	0.76 ± 0.09
Phosphatidylinositol	1.05 ± 0.07	0.99 ± 0.11

[a]Results are expressed as μg of lipid phosphorous/10^6 cells and are means ± SEM for six determinations. Statistical significances of differences from the control are indicated by [a]$P \leq 0.015$.

mediates pools in the phosphatidylcholine biosynthetic pathway (19,20). So, Kent *et al.* (20) have demonstrated that when electropermeabilized cells are incubated with radiolabeled phosphocholine or CDP-choline there is no incorporation of radioactivity into phosphatidylcholine, therefore there is no mixing with the endogenous pool of these metabolic intermediates. This indicates that all three water-soluble reaction intermediates of phosphatidylcholine biosynthesis are not freely diffusible in the cell, but are channeled in some maner, resulting in a functional linkage between the individual steps in phosphatidylcholine biosynthesis. Thus, assuming that exogenous and endogenous precursors are not uniformly mixed, in the present study we have used exogenous substrates such as isotopically labelled serine, ethanolamine, or choline in order to analyze the individual steps in the biosynthesis of the different phospholipids.

With regard to the incorporation of serine into phospholipids, the results in Table 2 show that in control hepatocytes the label from this amino acid is found in phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine, while no detectable radioactivity exhibited sphingomyelin or phosphatidylinositol. Interestingly, in chronically ethanol-treated hepatocytes, an increase in the radioactivity associated with the three phospholipids can be seen, the increase being clearly higher in phosphatidylserine. Our results therefore indicate that chronic ethanol consumption markedly enhances the synthesis of phosphatidylserine from serine, producing an increase in the radioactive label in phosphatidylserine and consequently in the other phospholipids synthesized from it.

Phosphatidylserine is synthesized in mammals exclusively from a base-exchange reaction catalyzed by phosphatidylserine synthase, and it has indeed been reported that free fatty acids exert a marked activator effect on this enzyme activity in both liver (21) and brain (22) microsomes. Since cytosolic fatty acids significantly increase in the liver as a consequence of ethanol metabolism (6), they could be responsible in part for the increase in the phosphatidylserine synthase activity and thus in the synthesis of all serine-derived phospholipids found in this study. In addition, it has been consistently demonstrated in our (23) and other (24) laboratories that ethanol ingestion induces marked changes in the lipid composition and fluidity of hepatic microsomal membranes in chronic alcoholism, and since phosphatidylserine synthase is an enzyme located in the endoplasmic reticulum, it is possible that the modifications induced by ethanol in this membrane may also be responsible for the alterations in this enzyme activity.

Similarly to that found in phosphatidylserine synthesis, hepatocytes from chronically ethanol-treated rats exhibit a higher capacity to synthesize phosphatidylethanolamine from exogenous ethanolamine (Table 3). In fact, in the alcoholic group, the incorporation of ethanolamine was three times higher than that found in control hepatocytes. As a consequence of this increase, phosphatidylcholine and the methylated intermediates, phosphatidyl-*N*-monomethylethanolamine, and phosphatidyl-*N,N*-dimethylethanolamine also show a higher radioactive label in ethanol-treated hepatocytes. An increase in the lipogenic activity in rat livers after ethanol administration has been observed by different authors, and it has been related to an increase in phosphatidate phosphohydrolase activity (25,26). Thus, the enhancement in the synthesis of phosphatidylethanolamine found in our study is consistent with an activation in phosphatidate phosphohydrolase activity, which could produce an increase in the availability of diacylglycerol substrate necessary for the last step in phosphatidylethanolamine biosynthesis catalyzed by the enzyme ethanolamine phosphotransferase. In addition, it is interesting to note that the increase in the synthesis of phosphatidylethanolamine is accompanied by a significant decrease in the radioactivity of the metabolic intermediates, ethanolamine phosphate and CDP-ethanolamine (Table 3).

As far as the influence of ethanol consumption on the biosynthesis of phosphatidylcholine *via* CDP-choline is concerned, the results shown in Table 4 indicate that in hepatocytes isolated from chronically ethanol-treated rats the incorporation of choline into phosphatidylcholine decreases very significantly, although neither choline phosphate nor CDP-choline shows any alteration in their radioactive labels. Thus, this inhibition of phosphatidylcholine biosynthesis may be attributed to the specific action of ethanol on choline phosphotransferase activity, involved in the final step of phosphatidylcholine synthesis. Other authors have also found a significant decrease in phosphatidylcholine synthesis in the livers of chronically ethanol-treated rats (8), suggesting that this effect might be due to an acute depletion of the levels of the diacyl-

TABLE 2
Effect of Chronic Ethanol Consumption on the Incorporation of Radioactivity from [3-^{3}H]Serine into Different Phospholipids in Isolated Hepatocytes[a]

	Control	Ethanol
Phosphatidylcholine	0.52 ± 0.02	0.79 ± 0.08[a]
Phosphatidylethanolamine	0.28 ± 0.02	0.48 ± 0.02[b]
Phosphatidylserine	1.79 ± 0.05	4.45 ± 0.19[b]

[a]Results are expressed as pmol/min × 10^6 cells and are means ± SEM for four determinations. Statistical significances of differences from the control are indicated by [a]$P \leq 0.035$; [b]$P \leq 0.005$.

TABLE 3
Effect of Chronic Ethanol Consumption on the Incorporation of Radioactivity from [1-^{3}H]Ethanolamine into Metabolic Intermediates of the Phosphatidylethanolamine Biosynthesis and Their Methylated Derivatives in Isolated Hepatocytes[a]

	Control	Ethanol
Ethanolamine phosphate	14.15 ± 0.31	3.87 ± 0.29[b]
CDP-ethanolamine	0.91 ± 0.12	0.59 ± 0.01[a]
Phosphatidylethanolamine	13.91 ± 0.14	41.95 ± 0.95[b]
N-methylphosphatidylethanolamine	3.28 ± 0.22	2.70 ± 0.16
N,N-dimethylphosphatidylethanolamine	0.16 ± 0.01	0.40 ± 0.03[a]
Phosphatidylcholine	0.49 ± 0.04	1.43 ± 0.05[b]

[a]Results are expressed as pmol/min × 10^6 cells and are means ± SEM for four determinations. Statistical significances of differences from the control are indicated by [a]$P \leq 0.04$; [b]$P \leq 0.0001$.

TABLE 4
Effect of Chronic Ethanol Consumption on the Incorporation of Radioactivity from [methyl-^{14}C]Choline into Betaine and Choline-Containing Metabolites in Isolated Hepatocytes[a]

	Control	Ethanol
Betaine	29.32 ± 3.27	7.62 ± 1.43[a]
Choline phosphate	37.52 ± 1.07	38.87 ± 0.66
CDP-choline	0.86 ± 0.07	0.72 ± 0.04
Phosphatidylcholine	97.28 ± 2.3	573.49 ± 1.43[b]

[a]Results are expressed as pmol/min × 10^6 cells and are means ± SEM for four determinations. Statistical significances of differences from the control are indicated by [a]$P \leq 0.001$; [b]$P \leq 0.0001$.

glycerol substrate required for phosphatidylcholine biosynthesis. Nevertheless, both phosphatidylcholine and phosphatidylethanolamine synthesis *via* CDP-derivatives requires diacylglycerol, and so a reduction in the diacylglycerol levels should affect these pathways similarly. Since as mentioned above (Table 3), the radioactive label of phosphatidylethanolamine from [1-^{3}H]ethanolamine is clearly enhanced after ethanol treatment while that of phosphatidylcholine is significantly reduced, it is evident that the changes in the diacylglycerol levels cannot be responsible for the decrease in the phosphatidylcholine biosynthesis, at least under our experimental conditions.

To sum up, the comparative analysis of the biosynthesis of both phosphatidylcholine and phosphatidylethanolamine *via* CDP-derivatives allows us to demonstrate that ethanol ingestion exerts a differential effect on the Kennedy pathway, an increase in phosphatidylethanolamine synthesis, and a decrease in the biosynthesis of phosphatidylcholine. In any case, the lower phosphatidylcholine synthesis is consistent with the reduction in the level of this phospholipid found in our study. The increase in the phosphatidylethanolamine and phosphatidylserine synthesis observed in alcoholic rats when ethanolamine or serine is used as exogenous precursor does not suppose an increase in the levels of these phospholipids after 31 d of ethanol ingestion (Table 1). This could be explained as a result of a higher methylation of phosphatidylethanolamine, synthesized from exogenous serine or ethanolamine, to give phosphatidylcholine in order to compensate its decrease by the CDP-choline pathway.

On the other hand, it can also be observed in Table 4 that the radioactivity from choline appears as well in betaine, the end-product of their oxidation by choline oxidase. Remarkably, in the ethanol group, the radioactivity in betaine is clearly lower than that found in control hepatocytes. Although this reduction could be explained as the result of a specific inhibition of choline oxidase, there is experimental evidence in chronic alcoholism to suggest an enhancement in the use of betaine (27). In fact, the decrease in the radioactive label of betaine found in our study after chronic ethanol ingestion is consistent with a compensatory mechanism by which betaine is preferably used as methyl donor in situations of impairment in the methionine synthesis to allow the maintenance of the hepatic *S*-adenosylmethionine levels. So, other authors have demonstrated an increase in the betaine–homocysteine methyltransferase activity in chronic alcoholism (28), which agrees with our data. These results support the notion that betaine exerts a hepatoprotective effect due to its ability to stimulate the formation of *S*-adenosylmethionine in the liver.

All these results are of a great physiological interest because phosphatidylcholine and phosphatidylethanolamine are structural and/or functional components of membranes and lipoproteins and thus their formation and/or metabolism is required for signal transduction, membrane formation, and lipoprotein synthesis. Clearly any change in their metabolism could alter these processes contributing to the pathology associated with alcoholism.

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Dietary Eritadenine-Induced Alteration of Molecular Species Composition of Phospholipids in Rats

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ABSTRACT: The effect of dietary eritadenine, a hypocholesterolemic compound found in the mushroom *Lentinus edodes*, on the fatty acid and molecular species profiles of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in the plasma and liver microsomes was investigated in relation to the hypocholesterolemic action of the compound in rats. Animals were fed the control or eritadenine-supplemented (50 mg/kg diet) diet for 14 d. Eritadenine supplementation significantly decreased the plasma concentrations of cholesterol and phospholipids, but not triglycerides. The PC/PE ratio of liver microsomes, but not plasma, was also markedly decreased by eritadenine. Eritadenine supplementation was found to increase the proportion of 18:2n-6 and, inversely, to decrease the proportion of 20:4n-6 and 22:5n-6 in plasma PC and liver microsomal PC and PE, indicating that eritadenine depressed the metabolism of linoleic acid. The effect of eritadenine on the profile of n-3 fatty acids was dissimilar in PC and PE. These changes in fatty acid composition were selectively reflected in the molecular species composition of both PC and PE; the extent of increase in 16:0-18:2 molecular species or decrease in 18:0-20:4 molecular species was apparently greater than that of other molecular species containing 18:2 or 20:4 in the sn-2 position. These results suggest that, in addition to the decrease in liver microsomal PC/PE ratio, the alteration of plasma PC molecular species composition might also participate in the hypocholesterolemic action of eritadenine.

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The mushroom *Lentinus edodes* (Shiitake in Japanese) has a potent hypocholesterolemic action when fed to rats (1), and eritadenine [2(R),3(R)-dihydroxy-4-(9-adenyl)-butyric acid] was isolated from the mushroom as a hypocholesterolemic factor (2,3). Eritadenine also exists in another mushroom *Agaricus bisporus* (champignon) to a lesser extent (4). To date, a number of studies have reported on the mechanism by which *L. edodes* or eritadenine exerts its hypocholesterolemic action, but the detailed mechanism is not yet fully elucidated.

Recently, we provided evidence that the hypocholesterolemic action of *L. edodes* (5) or eritadenine (6,7) might be elicited through a decrease in the ratio of phosphatidylcholine (PC) to phopshatidylethanolamine (PE) in liver microsomes of rats. This eritadenine-induced alteration of hepatic phospholipid class profile was found to arise primarily from the depression of PE *N*-methylation (6). However, it is still unclear how the decreased PC/PE ratio of liver microsomes is associated with the reduction of plasma cholesterol. One of the interesting reports concerned with the relationship between phospholipid metabolism and the regulation of plasma cholesterol is that the difference in the molecular species of plasma high density lipoprotein (HDL) PC could largely influence the uptake of HDL cholesterol by tissues (8,9). These findings led us to postulate a working hypothesis that eritadenine may elicit its hypocholesterolemic effect not only by decreasing the PC/PE ratio of liver microsomes, but also by altering the molecular species composition of plasma lipoprotein PC. However, no information currently exists concerning eritadenine's ability to modify the molecular species composition of plasma lipoprotein PC.

In this study, therefore, the effect of dietary eritadenine on the profiles of fatty acids and molecular species of plasma and liver microsomal major phospholipids (PC and PE) was investigated with the aim of clarifying the mechanism of the hypocholesterolemic action of eritadenine in rats.

MATERIALS AND METHODS

Animals. Twelve male 5-wk-old rats of the Wistar strain were received at 90–100 g body weight from Japan SLC (Hamamatsu, Japan). The rats were individually housed in hanging stainless-steel wire cages kept in an isolated room at a controlled temperature (23–25°C) and humidity (40–60%). Lights were maintained on a 12-h cycle (lights on from 0600 to 1800 h). For 5 d, animals were fed the powdered laboratory stock diet, which resembled the control diet described below except that sucrose and lactose were replaced by starch and 2 g/kg diet of choline chloride was added. Then, they were divided into two groups of six rats each with similar mean weights (~121 g) and allowed free access to the experimental diets and water for 14 d.

Diets. Two diets, control and eritadenine-supplemented, were utilized in this study. The control diet consisted of the following (g/kg): casein (Nacalai tesque, Kyoto, Japan), 250;

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Abbreviations: GLC, gas–liquid chromatography; HDL, high density lipoprotein; HPLC, high-performance liquid chromatography; LDL, low density lipoprotein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SAH, *S*-adenosylhomocysteine; TLC, thin-layer chromatography; VLDL, very low density lipoprotein.

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cornstarch (Fuji-seifun, Shimizu, Japan), 450; sucrose (Fuji-seito, Shimizu, Japan), 200; corn oil (Honen, Tokyo, Japan), 50; AIN-76 mineral mixture (Oriental Yeast, Tokyo, Japan), 35; AIN-76 vitamin mixture (Oriental), 10; choline chloride (Wako Pure Chemical, Osaka, Japan), 4; lactose (Wako), 1. Eritadenine, which was isolated from dried *L. edodes* mushroom according to the method of Tokita *et al.* (10), was fully mixed with lactose and added to the diet at a level of 50 mg/kg diet at the expense of lactose. This dose level was proven to be sufficient to elicit maximal hypocholesterolemic effect (6). Choline chloride was introduced into the diet at a relatively high level (4 g/kg) because lower levels of dietary choline led to the development of fatty livers (7). The fatty acid composition of corn oil used was as follows (wt%): 16:0, 12.2; 18:0, 1.8; 18:1n-9, 32.8; 18:2n-6, 51.4; and 18:3n-3, 1.6.

Tissue collection and fractionation. At the end of the feeding period, rats were killed by decapitation under light anethesia with diethyl ether between 1100 and 1200 h and without prior food deprivation. Plasma was separated from heparinized whole blood by centrifugation at 2000 × *g* for 20 min at 4°C. An aliquot of the plasma was stored at 4°C until subsequent analyses for plasma lipid concentrations, and the residual plasma was stored at −80°C until analyses for phospholipids. After collection of blood, the whole liver was quickly removed, rinsed in ice-cold saline, blotted on filter paper, and cut into two portions. One portion of the liver was quickly frozen in liquid nitrogen and stored at −30°C until analyses for liver lipid concentrations. The other portion was homogenized in 4 vol (vol/wt) of an ice-cold 10 mM Tris-HCl buffer (pH 7.4) containing 0.15 M KCl. The homogenates were centrifuged at 10,000 × *g* for 10 min at 4°C, and the resultant supernatants were further centrifuged at 105,000 × *g* for 60 min at 4°C to obtain the microsomal fraction as a precipitate. The microsomes obtained were resuspended in the homogenizing buffer and stored at −80°C until analyses for phospholipids.

Lipid analysis. The plasma concentrations of total cholesterol, HDL-cholesterol, free cholesterol, triglycerides, and phospholipids were measured enzymatically with kits (Wako). The difference between total cholesterol and HDL cholesterol or free cholesterol was assumed to be very low density lipoprotein (VLDL) + low density lipoprotein (LDL) cholesterol or esterified cholesterol, respectively. The lipids of liver, liver microsomes and plasma were extracted by the method of Folch *et al.* (11). The cholesterol, triglycerides and phospholipids in the extract of liver were measured by the methods of Zak (12), Fletcher (13), and Bartlett (14), respectively. For the determination of phospholipid class composition, the phospholipids in the extracts of liver microsomes and plasma were separated into each class by thin-layer chromatography (TLC) on Silica Gel 60 (E. Merck, Darmstadt, Germany) using chloroform/methanol/water (65:25:4, by vol) as a developing solvent. The bands of each phospholipid class were visualized in iodine vapor, scraped off the plate, and analyzed directly for inorganic phosphorus (14). For the determinations of fatty acids and molecular species, PC and PE were similarly separated by TLC, visualized with dichlorofluorescein, scraped off the plate and extracted with chloroform/methanol (1:1, vol/vol). Aliquots of PC and PE were treated with 14% (wt/wt) BF_3/methanol reagent (Wako) (15), and fatty acid methyl esters formed were analyzed by gas–liquid chromatography (GLC) on a Model GC-17A (Shimadzu Seisakusho, Kyoto, Japan), equipped with a TC-FFAP capillary column (0.25 mm × 30 m; GL Sciences, Tokyo, Japan). Other aliquots of PC and PE were converted to diacylglycerol benzoates according to the method of Blank *et al.* (16). The diacylglycerol benzoates were separated into each molecular species by high-performance liquid chromatography (HPLC) on a Model LC-6A system (Shimadzu), equipped with an ODS column (4.6 × 250 mm; Lichrosorb RP-18; E. Merck), essentially according to the method of Blank *et al.* (16). Identification of molecular species was made by both comparison with the elution times of some authentic molecular species and fatty acid analysis of eluents by GLC. Because some peaks consisted of two molecular species, the ratio of two molecular species was estimated by the analysis of fatty acids by GLC. Figure 1 shows a typical HPLC chromatogram.

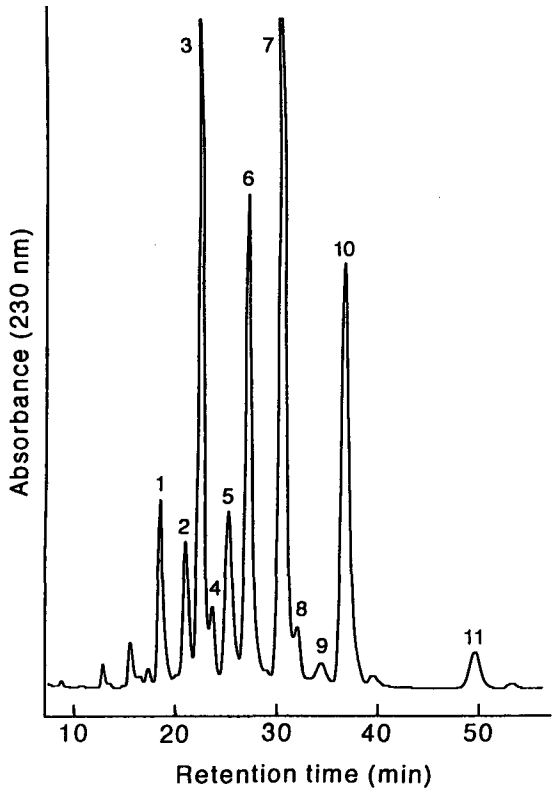


FIG. 1. A typical high-performance liquid chromatography chromatogram of liver microsomal phosphatidylcholine molecular species in rats. Peak numbers 1–11 indicate the following molecular species: 1, 16:0-22:6; 2, 18:1-20:4; 3, 16:0-20:4; 4, 16:0-22:5; 5, 18:1-18:2 + 18:0-22:6; 6, 16:0-18:2; 7, 18:0-20:4; 8, 18:0-22:5; 9, 18:1-18:1; 10, 16:0-18:1 + 18:0-18:2; 11, 18:0-18:1.

Statistical analysis. Data were expressed as mean ± SEM for six rats. Student's *t*-test was used to examine the signifi-

cance between control and eritadenine-supplemented groups. Simple correlation between variables was calculated by linear regression analysis.

RESULTS

The body weight gain, food intake and relative liver weight of rats were not significantly different between the two dietary groups (Table 1).

Liver and plasma lipid concentrations. The concentrations of cholesterol, triglycerides, and phospholipids in the liver were slightly, but significantly, increased by eritadenine supplementation. The plasma cholesterol concentrations of total, VLDL + LDL, HDL, free, and esterified were all significantly decreased by eritadenine supplementation. Although the plasma phospholipid concentration was also significantly decreased, the plasma triglyceride concentration was unaffected by eritadenine.

Phospholipid class composition. Table 2 shows the effect of dietary eritadenine on the phospholipid class composition of both liver microsomes and plasma. Eritadenine supplementation significantly decreased the proportion of PC and, inversely, increased the proportion of PE in liver microsomes. Consequently, the PC/PE ratio was significantly decreased by eritadenine. In contrast, there was no difference in the concentration of total phospholipids in liver microsomes between the control and eritadenine-supplemented groups (405 ± 12 vs. 416 ± 9 nmol/mg protein). In the plasma, the proportion of PC was slightly decreased by eritadenine supplementation, but neither the proportion of PE nor the PC/PE ratio was significantly affected by eritadenine.

Fatty acid composition of phospholipids. Table 3 shows the effect of dietary eritadenine on the fatty acid composition of plasma PC and liver microsomal PC and PE. Eritadenine supplementation significantly increased the proportion of 18:2n-6 and, inversely, decreased the proportion of 20:4n-6 and 22:5n-6 in both PC and PE. Consequently, the ratio of 20:4n-6 + 22:5n-6 to 18:2n-6 was markedly decreased by eritadenine. Eritadenine supplementation also significantly increased the proportion of 16:0 and decreased the proportion of 18:0 in plasma and liver microsomal PC, although these changes were relatively minor in liver microsomal PE. Eritadenine supplementation significantly increased the proportion of n-3 fatty acids in liver microsomal PE, unlike in plasma and liver microsomal PC.

Molecular species composition of phospholipids. Table 4 shows the effect of dietary eritadenine on the molecular species composition of plasma PC and liver microsomal PC and PE. The proportion of 16:0-18:2 PC was markedly increased by eritadenine supplementation, whereas the proportion of 18:0-18:2 PC or 18:1-18:2 PC was only slightly affected by eritadenine. Similarly, the eritadenine-induced decrease in 20:4n-6 of PC was more prominently reflected in the decrease in 18:0-20:4 PC than in the decrease in 16:0-20:4 PC or 18:1-20:4 PC. The eritadenine-induced alteration of 16:0-18:2 and 18:0-20:4 molecular species was also observed in liver microsomal PE, although the extent of the change was smaller than that in PC.

TABLE 1
Effect of Dietary Eritadenine on Plasma and Liver Lipid Concentrations in Rats[a]

	Dietary group	
	Control	+Er
Body weight gain (g/14 d)	81 ± 2	82 ± 1
Food intake (g/14 d)	188 ± 3	184 ± 2
Liver weight (g)	9.6 ± 0.3	9.5 ± 0.1
Liver lipids (μmol/g liver)		
Cholesterol	8.6 ± 0.2	10.2 ± 0.4[c]
Triglycerides	31.0 ± 1.5	43.9 ± 3.4[b]
Phospholipids	30.1 ± 0.3	32.7 ± 0.4[d]
Plasma lipids (mmol/L)		
Total cholesterol	2.83 ± 0.06	1.34 ± 0.01[d]
VLDL+LDL cholesterol	1.47 ± 0.06	0.75 ± 0.02[d]
HDL cholesterol	1.36 ± 0.05	0.60 ± 0.02[d]
Free cholesterol	0.89 ± 0.04	0.57 ± 0.02[d]
Esterified cholesterol	1.94 ± 0.07	0.77 ± 0.02[d]
Triglycerides	2.45 ± 0.12	2.57 ± 0.14
Phospholipids	2.95 ± 0.06	1.97 ± 0.04[d]

[a]Values are mean ± SEM for six rats. [b,c,d]A significant difference from the value of control group at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. Abbreviations: Er, eritadenine; VLDL, very low density lipoprotein; HDL, high density lipoprotein; LDL, low density lipoprotein.

TABLE 2
Effect of Dietary Eritadenine on Phospholipid Class Composition of Liver Microsomes and Plasma in Rats[a]

	Dietary group	
	Control	+Er
Liver microsomes (mol%)		
PC	61.3 ± 0.3	49.4 ± 0.4[d]
PE	17.5 ± 0.2	30.3 ± 0.2[d]
PS + PI	12.2 ± 0.4	12.1 ± 0.2
SM	5.5 ± 0.1	4.8 ± 0.4
Others	3.5 ± 0.2	3.4 ± 0.1
PC/PE	3.5 ± 0.0	1.6 ± 0.0[d]
Plasma (mol%)		
PC	60.7 ± 1.0	56.1 ± 0.4[c]
PE	6.4 ± 0.3	6.2 ± 0.2
PS + PI	3.7 ± 0.1	4.2 ± 0.1[c]
SM	5.7 ± 0.2	7.1 ± 0.2[d]
LPC	19.6 ± 0.6	20.0 ± 0.6
Others	4.0 ± 0.3	6.5 ± 0.3[d]
PC/PE	9.6 ± 0.6	9.1 ± 0.4

[a]Values are mean ± SEM for six rats. [c,d]A significant difference from the value of control group at $P < 0.01$ and $P < 0.001$, respectively. Abbreviations: Er, eritadenine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; LPC, lysophosphatidylcholine.

DISCUSSION

Effect on phospholipid class composition. It has been shown that eritadenine is a potent inhibitor of *S*-adenosylhomocysteine (SAH) hydrolase (17) and consequently increases the concentration of SAH in isolated hepatocytes (18) and intact

TABLE 3
Effect of Dietary Eritadenine on the Fatty Acid Composition of Plasma and Liver Microsomal Phospholipids in Rats[a]

Fatty acid	Plasma PC		Liver microsomal PC		Liver microsomal PE	
	Control	+Er	Control	+Er	Control	+Er
	g/100 g fatty acids					
16:0	22.0 ± 0.4	29.3 ± 0.3[d]	19.3 ± 0.3	23.8 ± 0.3[d]	19.4 ± 0.4	20.1 ± 0.2
18:0	21.8 ± 0.3	14.1 ± 0.4[d]	18.2 ± 0.3	13.6 ± 0.6[d]	23.3 ± 0.4	22.1 ± 0.3[b]
18:1n-9	8.1 ± 0.3	10.9 ± 0.2[d]	12.0 ± 0.2	12.6 ± 0.3	5.8 ± 0.1	5.9 ± 0.2
18:2n-6	13.8 ± 0.2	27.0 ± 0.5[d]	11.9 ± 0.2	23.3 ± 0.6[d]	2.9 ± 0.1	7.3 ± 0.1[d]
20:4n-6	25.9 ± 0.4	13.2 ± 0.4[d]	27.8 ± 0.3	18.6 ± 0.7[d]	27.7 ± 0.3	24.4 ± 0.3[d]
22:5n-6	2.7 ± 0.1	0.2 ± 0.0[d]	2.5 ± 0.1	0.2 ± 0.0[d]	6.0 ± 0.4	1.2 ± 0.2[d]
22:5n-3	0.5 ± 0.0	0.4 ± 0.0[b]	0.4 ± 0.0	0.3 ± 0.0[b]	0.9 ± 0.1	1.7 ± 0.1[d]
22:6n-3	3.4 ± 0.1	1.4 ± 0.1[d]	3.8 ± 0.1	2.2 ± 0.1[d]	10.4 ± 0.1	15.3 ± 0.2[d]
Σn-6/18:2	2.1 ± 0.1	0.5 ± 0.0[d]	2.5 ± 0.1	0.8 ± 0.0[d]	11.6 ± 0.4	3.5 ± 0.1[d]

[a]Values are mean ± SEM for six rats. [b,d]A significant difference from the value of control group at $P < 0.05$ and $P < 0.001$, respectively. Abbreviations: Er, eritadenine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Σn-6, sum of 20:4n-6 and 22:5n-6.

livers (6) of rats. This increase in hepatic SAH concentration caused by eritadenine was thought to bring about a depression of PE *N*-methylation. Actually, the *in vivo* incorporation of the labeled methyl group of methionine into plasma and liver microsomal PC was almost completely inhibited in rats fed a diet supplemented with eritadenine (50 mg/kg diet) (6). These results, together with results on the analysis of hepatic phospholipid precursors, indicated that dietary supplementation with eritadenine led to a decrease in the liver microsomal PC/PE ratio primarily through depressed PE *N*-methylation and secondarily through increased PE biosynthesis *via* the CDP-ethanolamine pathway (6,7). The present study confirmed that eritadenine can markedly decrease the PC/PE ratio of liver microsomes. In contrast, the PC/PE ratio of plasma was found not to be affected by eritadenine, supporting the idea that the phospholipid class composition of plasma is resistant to dietary manipulation compared with that of liver microsomes (19).

Effect on fatty acid and molecular species composition. The present study clearly demonstrated that dietary supplementation with eritadenine could cause a drastic alteration of fatty acid composition and molecular species composition not only of liver microsomal PC, but also of plasma PC. The results of fatty acid analysis of phospholipids indicate that the metabolic conversion of 18:2n-6 to 20:4n-6 or 22:5n-6 was largely depressed in rats fed the eritadenine-supplemented diet. Although the activity of enzymes participating in the metabolism of fatty acids was not measured in the present study, another study by our laboratory showed that the Δ6-desaturase activity of liver microsomes was markedly depressed in eritadenine-treated rats (unpublished observation). This suggests that eritadenine depresses the metabolism of linoleic

TABLE 4
Effect of Dietary Eritadenine on the Molecular Species Composition of Plasma and Liver Microsomal Phospholipids in Rats[a]

Molecular species	Plasma PC		Liver microsomal PC		Liver microsomal PE	
	Control	+Er	Control	+Er	Control	+Er
	mol/100 mol					
16:0–18:1	6.7 ± 0.3	6.4 ± 0.4	7.6 ± 0.4	8.6 ± 0.2[b]	1.7 ± 0.1	3.0 ± 0.1[d]
18:0–18:1	1.2 ± 0.2	0.6 ± 0.1[c]	1.9 ± 0.1	1.3 ± 0.1[c]	0.4 ± 0.0	0.2 ± 0.0[d]
18:1–18:1	1.3 ± 0.1	1.0 ± 0.1	1.9 ± 0.4	1.3 ± 0.1	0.6 ± 0.1	0.3 ± 0.0[b]
16:0–18:2	18.6 ± 1.1	47.3 ± 0.9[d]	6.8 ± 0.4	27.4 ± 0.5[d]	5.3 ± 0.2	11.0 ± 0.3[d]
18:0–18:2	10.0 ± 0.5	9.6 ± 0.5	11.4 ± 0.5	13.1 ± 0.3[b]	2.7 ± 0.1	4.6 ± 0.1[d]
18:1–18:2	3.7 ± 0.1	4.2 ± 0.2[b]	4.0 ± 0.2	4.9 ± 0.1[c]	2.1 ± 0.0	3.6 ± 0.1[d]
16:0–20:4	18.1 ± 0.9	10.4 ± 0.4[d]	18.4 ± 0.5	14.5 ± 0.4[d]	15.8 ± 0.1	15.5 ± 0.3
18:0–20:4	17.7 ± 1.0	3.2 ± 0.2[d]	26.3 ± 0.2	10.5 ± 0.3[d]	31.1 ± 0.6	23.4 ± 0.4[d]
18:1–20:4	5.5 ± 0.3	6.0 ± 0.5	5.3 ± 0.3	7.6 ± 0.4[d]	7.0 ± 0.3	7.0 ± 0.1
16:0–22:5	3.4 ± 0.2	1.1 ± 0.1[d]	3.1 ± 0.2	0.6 ± 0.0[d]	8.3 ± 0.3	2.7 ± 0.1[d]
18:0–22:5	1.9 ± 0.1	0.4 ± 0.0[d]	3.5 ± 0.0	0.3 ± 0.0[d]	4.0 ± 0.2	2.5 ± 0.1[d]
16:0–22:6	5.2 ± 0.3	2.6 ± 0.3[d]	4.5 ± 0.1	4.1 ± 0.2	12.8 ± 0.4	14.2 ± 0.3[b]
18:0–22:6	2.5 ± 0.1	2.8 ± 0.1[b]	2.6 ± 0.1	3.1 ± 0.1[c]	4.8 ± 0.1	8.4 ± 0.2[d]

[a]Values are mean ± SEM for six rats. [b,c,d]A significant difference from the value of control group at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. Abbreviations: Er, eritadenine; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

acid by decreasing the activity of Δ6-desaturase, a rate-limiting step for linoleic acid metabolism (20). Further, there are lines of indirect evidence that the decrease in liver microsomal PC/PE ratio may be associated with the decrease in Δ6-desaturase activity. In addition to eritadenine, certain hypocholesterolemic substances such as PE (21), ethanolamine (21,22), glycine (22), and some dietary proteins including soybean protein (19) have been shown to decrease the PC/PE ratio of liver microsomes in rats. Of these substances, PE or ethanolamine (23) and soybean protein (24) were shown to bring about a decrease in the 20:4/18:2 ratio of plasma and liver microsomal PC accompanying a decrease in the activity of microsomal Δ6-desaturase. Further, it was shown that a decrease in the PC/PE ratio caused by choline deficiency (25) or vitamin B_6-deficiency (26) was coupled with a decrease in the activity of Δ5- or Δ6-desaturase, respectively, in liver microsomes of rats. These results indicate that the phospholipid class profile of liver microsomes, as represented by the PC/PE ratio, is one of the factors which affect the activity of desaturases (27,28). Taking these findings into consideration, it is possible that the depression of fatty acid metabolism by eritadenine is also mediated by a decrease in the PC/PE ratio of liver microsomes.

It seems reasonable to consider that the molecular species composition of plasma PC reflects, to some extent, that of liver microsomal PC, because the greatest part (*ca.* 80%) of plasma PC is derived from the liver (29). In fact, as a whole, there was a similarity of PC molecular species composition between plasma and liver microsomes when analyzed using values listed in Table 4 ($r = 0.842$, $P < 0.001$), although there was an apparent divergence in the proportion of specific molecular species such as 16:0-18:2 and 18:0-20:4. This divergence may be accounted for in part by the fact that certain molecular species, e.g., 18:0-20:4, were relatively not secreted from the liver as a lipoprotein component (30). Further, plasma HDL phospholipids are known to undergo deacylation by lecithin:cholesterol acyltransferase with a substrate specificity for molecular species. It is thought that phospholipid molecular species composition is regulated by a complex mechanism, e.g., biosynthesis of whole phospholipid molecule *via* different pathways, and subsequent remodeling by deacylation-reacylation. In addition, the depression of linoleic acid metabolism appears to contribute to the eritadenine-induced alteration of phospholipid molecular species which contain n-6 fatty acids in the *sn*-2 position, although this cannot account for the selective alteration of molecular species composition. In the rat liver, PC is deduced to be synthesized *via* the CDP-choline pathway by 60–80% and *via* the PE *N*-methylation pathway by 20–40% (31). In contrast, it is thought that PC biosynthesis *via* the latter pathway is largely inhibited in rats fed a eritadenine-supplemented diet. Thus, eritadenine modifies the relative dependency of PC biosynthesis on the two major pathways in the liver. However, it is unlikely that the cessation of PC biosynthesis *via* the PE *N*-methylation itself is a main cause of eritadenine-induced selective alteration of PC molecular species composition because eritadenine increased 16:0-18:2 molecular species and decreased 18:0-20:4 molecular species in both liver microsomal PC and PE. It is unclear how eritadenine affects the remodeling of phospholipids.

The present study demonstrated that the level of n-3 fatty acids in liver microsomal PE were increased, whereas those in PC were decreased by eritadenine. This is other evidence that *N*-methylation of PE in the eritadenine-treated rats was not active, although the mechanism for the differential effect of eritadenine is currently unclear. Contrary to the results presented here, Samborski *et al.* (32) showed that the inhibition of PE *N*-methylation by 3-deazaadenosine, another inhibitor of SAH hydrolase, did not affect the PC or PE molecular species composition of cultured rat hepatocytes. One of the reasons for this discrepancy may be the difference in the experimental conditions employed, i.e., *in vivo* or cells in culture, and relatively long-term (14 d) or short-term (up to 12 h) treatment of the inhibitors. The data on the time-dependent *in vivo* effect of dietary eritadenine on the molecular species composition of phospholipids should help to explain the discrepancy.

Mechanism of hypocholesterolemic action. Previous studies have suggested that eritadenine exerts its hypocholesterolemic action through depressed secretion of lipoprotein cholesterol from the liver into the blood circulation and/or increased uptake of plasma lipoprotein cholesterol by tissues (33,34). *Lentinus edodes* mushroom or eritadenine indeed causes, in many cases, fat accumulation in the liver with overall decreases in plasma lipids in rats. However, this eritadenine-induced fatty liver and a decrease in plasma triglyceride concentration could be effectively prevented by elevating the level of dietary choline without diminution of the hypocholesterolemic action of eritadenine (7). This suggests that the essential hypocholesterolemic action of eritadenine cannot be ascribed to the depressed secretion of triglyceride-rich lipoprotein (VLDL) from the liver. In support of this, the present study also showed that eritadenine supplementation can significantly decrease the plasma concentrations of cholesterol and phospholipids without concomitant decrease in plasma triglyceride concentration.

In contrast, little information is available concerning the effect of eritadenine on the uptake of plasma lipoproteins by tissues. It is widely accepted that the uptake of plasma lipoproteins is regulated mainly by a receptor-mediated mechanism. On the other hand, it has been shown that the uptake of HDL by tissues was promoted by the hydrolysis of HDL phospholipids by hepatic lipase (35). Further, Kadowaki *et al.* (9) demonstrated that the uptake rate of cholesterol ester of reconstituted HDL by perfused rat livers was largely influenced by PC molecular species used for the reconstitution of HDL. They showed that the uptake of HDL cholesterol was most stimulated by 16:0-18:2 PC of five PC molecular species tested (16:0-18:2, 16:1-16:1, 18:0-18:2, 18:1-16:0, and 20:1-20:1). These findings suggest the possibility that certain types of hypocholesterolemic treatment to increase the proportion of specific PC molecular species, e.g., 16:0-18:2, of plasma HDL bring about a reduction of plasma cholesterol,

at least in part, through increased uptake of HDL cholesterol by tissues. This assumption, together with the results presented here that eritadenine supplementation drastically increased the proportion of 16:0-18:2 PC in the plasma, appears to support the idea that eritadenine might also exert its hypocholesterolemic action through the alteration of plasma PC molecular species composition. The direct evidence for this hypothesis, however, is yet to be presented.

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Intestinal Lymph Absorption of Butter, Corn Oil, Cod Liver Oil, Menhaden Oil, and Eicosapentaenoic and Docosahexaenoic Acid Ethyl Esters in Rats

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ABSTRACT: Adult male rats were surgically given a drainage catheter in the main mesenteric lymph duct. After an overnight fast, five groups of rats received intragastrically, in one bolus, butter, corn oil (CO), cod liver oil (CLO), menhaden oil (MO), or ethyl esters of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids (K80). Intestinal lymph was collected in these conscious animals, each hour during the first 6 h and in a single sample for the next 18 h. The absorption peak appeared earlier after MO and CO than after CLO administration. The quantities of triglycerides recovered during the first 6 h were significantly lower after butter (91 mg) and K80 (54 mg) administration than for the other three oils. No difference was observed between the vegetable oil and the two marine oils (CO = 173 mg, CLO = 148 mg, MO = 180 mg). The total triglyceride recovered in 24 h was highest after CLO (410 mg) and lowest with K80 (146 mg). An increase in the weight percentage of some characteristic fatty acids of the lipid mixtures was observed: oleic acid for butter, oleic and linoleic acids for CO, EPA and DHA for CLO, MO, and K80. Chylomicrons were the largest with CO, more numerous and smaller with CLO, and the smallest with K80. Results obtained illustrated the relation between gastrointestinal hydrolysis, enterocyte biochemical events, and lymph triglyceride absorption profiles as related to the composition and distribution of triglyceride fatty acids.

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Intestinal absorption (1–4) of dietary triglycerides, major lipid constituents of the lipid diet, is decisive for the biological availability of fatty acids of oils and fats. Intestinal bioavailability is a consequence of the fatty acid carbon number, of the degree of unsaturation and of reciprocal influences of the different fatty acids at different levels of the intestinal tract (2). The stomach is the site of an important action of a preduodenal lipase (5) and the initiation of emulsification (6,7). In the proximal small intestine, hydrolysis by the pancreatic lipase occurs, in association with the mixture of bile salts and pancreatic colipase. Whatever the hydrolysis rate, the specificity of lipases and the distribution of fatty acids on the glycerol backbone are decisive parameters (8,9), including the nature of the fatty acid, with the possibility of a slower hydrolysis of some polyunsaturated fatty acids (10,11). This point may explain some of the differences previously observed in intestinal absorption of triglycerides with similar fatty acid compositions but from varied origins, for example, land or marine animals (12,13). Mixed micelle formation follows and is obligatory for diffusion across the unstirred water layer to occur. Uptake of monomers of fatty acids and monoglycerides by enterocytes, although thought to be a passive diffusion process, might be facilitated by membrane fatty acid binding proteins (FABP) such as FABPpm (14) and a fatty acid translocase (15). In the enterocytes, I-FABPc and L-FABPc might facilitate the intracellular transport (16) and the activation and reesterification of fatty acids that precede the formation and exocytosis of chylomicrons and very low density lipoprotein (VLDL). Conflicting results in the literature relating to absorption recoveries of vegetable oils, land mammal fats, or marine oils might be reconciled on the basis of different experimental conditions, particularly modalities and/or level of administration (2,17–19,20–22).

The aim of this work is to compare intestinal absorption processes of four fats or oils representative of a spectrum of fatty acids, to understand some of the variations in intestinal absorption due to different fatty acid compositions. We chose butter, corn oil (CO), and cod liver oil (CLO) and menhaden oil (MO), two cold-water marine fish oils. In addition, we studied intestinal absorption of K80, a mixture of ethyl esters of eicosapentaenoic (EPA) and docosahexaenoic acid (DHA). For this purpose, we investigated recoveries and profiles of mesenteric lymph triglycerides and intestinal lymph lipoproteins, especially their morphological aspects and fatty acid composition.

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Abbreviations: CLO, cod liver oil; CO, corn oil; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FABP, fatty acid binding protein; GLC, gas–liquid chromatography; MO, menhaden oil; VLDL, very low density lipoprotein.

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MATERIALS AND METHODS

Animals. Official French regulations (n° 87848) for the care and use of laboratory animals were followed (n° 03056). Male Wistar rats weighing 180–250 g were obtained from Centre d'élevage Dépré (Saint Doulchard, France). They were housed for one week before the study in a controlled environment, with constant temperature and humidity and a dark period from 2000 to 0800 h. They were fed the standard nonpurified diet AO4 containing 3.5 g/100 g lipid (UAR Villemoisson sur Orge, Epinay sur Orge, France), and had free access to tap water.

Surgical procedure. A laparotomy was performed under ether anesthesia. Mesenteric lymph duct cannulation was performed using a heparinized polyethylene catheter (n°1, i.d. 0.3, o.d. 0.7; Biotrol, Paris, France) (23). Immediately after the surgery, rats were placed in restraining cages in an air-conditioned room (25°C). They did not receive solid food but had free access to a water solution containing sodium chloride (7 g/L) and potassium chloride (2 g/L).

Experimental procedures. The morning after surgery, basal lymph of fasting rats was collected for 1 h in tubes placed inside Dewar flasks containing ice, immediately before lipid administration. Then, 0.65 mL of slightly heated butter (above its melting point of 29°C), or 0.5 mL of the oils, or of K80 was administered intragastrically. The lymph was separately collected each hour during the first 6 h and in a single sample for the next 18 h from the conscious animals. According to the time period during lipid absorption, the lymph flow rate was two to three times as high as in the fasting state. In all cases, the animals whose lymph flow had not increased in this range were discarded (24). The samples were immediately treated for both morphological study of lipoproteins and lipid extraction followed by phospholipid, triglyceride, and fatty acid analysis.

Butter and oils. Table 1 shows the distribution of the major fatty acids of the butter and the three oils studied as measured by gas–liquid chromatography (GLC). Butter is particularly rich in medium-chain saturated fatty acids (C12:0, C14:0, and C16:0 represent 45% wt%). CO is characterized by the presence of C18 unsaturated fatty acids, especially oleic and linoleic acids, which represent 82.4% (wt%) of fatty acids. Marine fish oils constitute natural sources of n-3 polyunsaturated fatty acids. However, wide variations are recorded according to the species. While CLO contains monounsaturated very long chain fatty acids such as gadoleic (C20:1n-9) and cetoleic acids (C22:1n-11), MO provides only small traces of them, and is significantly enriched in both EPA (C20:5n-3, 16.4%) and DHA (C22:6n-3, 7%). In contrast to the fatty acid composition of butter and CO, the two marine oils span a wide range of fatty acids chain lengths from C_{14} to C_{22}. In some experiments with butter, 1.7×10^4 Bq of [1-^{14}C] oleic acid (specific activity: 2.1 GBq/mmol) was added before its gastric administration.

Lipids were purchased from Nu-Chek-Prep (Elysian, MN) or SIGMA-France (Saint Quentin Fallavier, France). The labeled fatty acid was provided by CEA (Gif sur Yvette, Saclay, France). Butter and CO were purchased at a local store. CLO was purchased at a local pharmacy, and MO was donated by the Zapata Protein Co. (Reedville, VA). Ethyl esters of EPA and DHA, K80, were generously supplied by Norsk Hydro, s.a. (N-0203; Oslo, Norway).

TABLE 1
Composition in Major Fatty Acids (wt%) of Administered Lipids[a]

Fatty acids	Butter	Corn oil	Cod liver oil	Menhaden oil	K80
4:0	4.0				
6:0	2.0				
8:0	1.0				
10:0	2.0				
12:0	4.0				
14:0	12.0	0.1	5.1	8.5	
16:0	29.0	13.2	12.5	21.4	
16:1	4.0		8.9	13.5	
16:4					0.3
18:0	11.0	2.9	3.3	3.5	
18:1n-5					0.1
18:1n-9	25.0	30.3	21.3	9.4	
18:2n-6	2.0	52.1	2.1	1.3	
18:3n-3	1.5	1.0	0.8	1.0	0.2
18:3n-6					0.2
18:4n-3			2.8	3.2	3.2
20:1n-9			2.5	1.4	
20:4n-6			0.4	1.1	1.6
20:4n-3				1.4	1.0
20:5n-3			9.6	16.4	53.2
21:5n-3					1.7
22:1n-11			6.3	0.2	
22:5n-3					3.2
22:6n-3			9.0	6.7	33.3

[a]Fatty acids of butter, corn oil, cod liver oil, and menhaden oil are in triglyceride chemical form. K80 is composed of ethyl esters.

Lipid analysis. Phospholipids and triglycerides were determined in lymph samples using enzymatic kits (Test-Combination Phospholipides-Test Peridichrom Triglycerides GPO-PAP; Boehringer, Mannheim, Germany). Lipids from the lymph of fasting rats (collected during the hour before the infusion of the lipids) and the intestinal lymph samples were extracted by dimethoxymethane–methanol (4:1, vol/vol) (25), dehydrated with absolute ethanol and then dissolved in chloroform. If necessary, the radioactivity of the total lipid extracts was determined when indicated using a liquid scintillation spectrophotometer (Packard Prias PLD Tricarb; Packard Instruments, Downers Grove, IL).

GLC analysis. The analysis of butter fatty acids was performed according to Gresti *et al.* (26); the analysis of K80 fatty acids was performed by Norsk Hydro. After extraction, CO, MO, and CLO fatty acids and fatty acids of collected lymph samples were methylated in the presence of boron trifluoride (27), with heptadecanoic acid as internal standard. Methyl esters were extracted using hexane and separated by GLC. These studies were carried out with a Packard chromatograph (model 417; Packard Instruments) and a capillary column (CP wax 52 CB 50 m; Chrompack, Les Ulis, France). Fatty acid analysis of lymph samples collected in fasting rats

or in rats after oil administration was performed at a constant temperature of 200°C with helium as a carrier gas. For butter experiments, the initial oven temperature was 130°C, followed by a temperature increase of 4°C/min until 225°C was reached. The peak areas were measured by an integrator (Chromjet; Spectra-Physics, San Jose, CA) and normalized to give the values as weight percentages of total fatty acids (g/100 g total fatty acids).

Microscopic observation of lymph particles. Lymph was defibrinized by gauze filtration and 10 µL fixed with 200 µL of osmium tetroxide (0.2 g/L) for 1 h at 4°C. One drop of fixed suspension was placed on a collodion carbon grid. After complete evaporation of the fixing solution, the particles were shadowed with gold-palladium at a 50° angle with an Edwards E12E vacuum evaporator (Edwards High Vacuum Limited, Crawlay, Sussex, United Kingdom) (28). Two or three grids of each lymph fraction were examined using a Hitachi HU 11E transmission electron microscope (Hitachi, Ltd., Tokyo, Japan). At least 1,000–2,000 shadowed particles for each lymph fraction were counted and classified by size.

Statistics. Results were expressed as micromole equivalents of labeled fatty acid when oleic acid was added in butter, using the specific activity of the labeled fatty acid added, and in milligrams of triglyceride or phospholipid for butter, oil and ethyl ester absorption studies. Values, expressed as means ± SEM, were examined by analysis of variance and significant differences between means from different fats or oils and across time within each fat or oil were tested by Student's *t*-test for an independent variable.

RESULTS

Mesenteric lymph triglyceride output. In the fasting state, mesenteric lymph triglycerides reached 21.2 ± 1.0 mg for a 6-h time period in the 26 rats of the experiments, and no significant difference was recorded between the lymph flows of the various groups (0.6 ± 0.1 mL/h). After lipid infusion, the lymph flow increased twofold, 1.1 ± 0.1 mL/h, regardless of the lipid type, with a maximum at the absorption peak of 1.74 ± 0.2 mL/h that was threefold higher than the fasting state. Table 2 summarizes lymph flows and lymph triglyceride outputs observed after intragastric administration of 0.65 mL of butter, 0.5 mL of CO, CLO, MO, or ethyl esters of EPA and DHA (K80).

The first 6 h of digestion and absorption after the intragastric administration of butter were accompanied by a lymph triglyceride output significantly lower ($P < 0.01$) than for the the three oils. No significant difference was observed in mesenteric lymph triglyceride outputs between the vegetable oil and the two marine oils. In the case of EPA and DHA in ethyl ester form, the amount of mesenteric lymph triglycerides was significantly lower than for the oils ($P < 0.001$) or in comparison with butter ($P < 0.001$).

Considered over a time period of 24 h, a significant difference was maintained between the amount of mesenteric lymph triglyceride output with the feeding of butter and CO, whereas all significant differences disappeared between the amounts of mesenteric lymph triglycerides recovered after the feeding of butter and the two marine oils. The lowest lymph triglyceride output was observed after administration of K80 ethyl esters.

TABLE 2
Lymph Flows and Lymph Triglyceride (TG) Outputs After Intragastric Administration of Different Lipids[a]

	Lymph flows[b]		TG outputs[c]	
Lipids studied	Fasting state (mL/h)	Absorption peak (mL/h)	TG, 0–6 h (mg)	TG, 0–24 h (mg)
Butter ($n = 6$)	0.65 ± 0.1[a]	1.1 ± 0.2[a]	91 ± 2[c]	346 ± 9[c]
Corn oil ($n = 4$)	0.70 ± 0.01[b]	2.0 ± 0.4[b]	173 ± 20[d]	282 ± 32[d]
Cod liver oil ($n = 6$)	0.30 ± 0.03[b]	1.3 ± 0.3[b]	148 ± 14[d]	410 ± 62[c,d]
Menhaden oil ($n = 6$)	0.70 ± 0.5[a]	2.2 ± 0.4[a]	180 ± 12[d]	347 ± 21[c,d]
K80 ($n = 4$)	0.75 ± 0.1[a]	2.1 ± 0.5[a]	54 ± 6[e]	146 ± 26[e]

[a]Values are means ± SEM for number of rats per group.
[b]Values are expressed in milliliters per hour for lymph flows in the fasting state and at the absorption peak and are horizontally significantly different ([a]$P < 0.05$, [b]$P < 0.025$).
[c]Values are expressed in milligrams for TG outputs. For the first 6 h of the experiments, means assigned different superscript letters (c,d,e) are vertically significantly different at $P < 0.01$ and at $P < 0.05$ for the following 18 h.

At all time periods, phospholipids represented only a minor contribution to the lymph lipids, i.e., of 6, 8, 12, 12, and 17% of the total in butter, CO, CLO, MO, and ethyl ester experiments, respectively.

Evaluated in triglyceride amounts vs. the quantities recovered over the time period of 24 h, 26 ± 1% and 32 ± 2% were recovered during the first 6 h in butter and ethyl ester experiments, while 62 ± 8% and 52 ± 6% were recovered with CO and MO experiments, respectively, and only 36 ± 6% with the CLO set of experiments.

Mesenteric lymph triglyceride profiles. The lymphatic triglyceride flux, expressed as milligrams per hour of triglycerides, was followed hour after hour during the first 6 h after lipid administration and then in a block from the 6th to the 24th hour (Fig. 1A). The lymphatic flux of CLO was observed to increase to a peak at 4.5 h and decline, whereas that of CO had reached a peak by the first measurement at 2 h and that of MO peaked prior to 1.5 h and ended by 6 h. Thus the maximum amount in lymph appeared early, as soon as the second hour, with butter and MO, the lipids richest in medium chain fatty acid triglycerides (49 and 43%, respectively, of $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, and $C_{16:1}$). CO exhibited a large peak amount in lymph which was spread over 3 h. In the case of CLO, rich in monounsaturated very long chain fatty acid triglycerides, the peak lymphatic absorption was delayed and narrow compared with those of the other two oils.

In accordance with triglyceride outputs, for the three oils, the rise in the lymphatic triglyceride flux gave equivalent amounts of triglycerides at the absorption peak (43.2 ± 4.2,

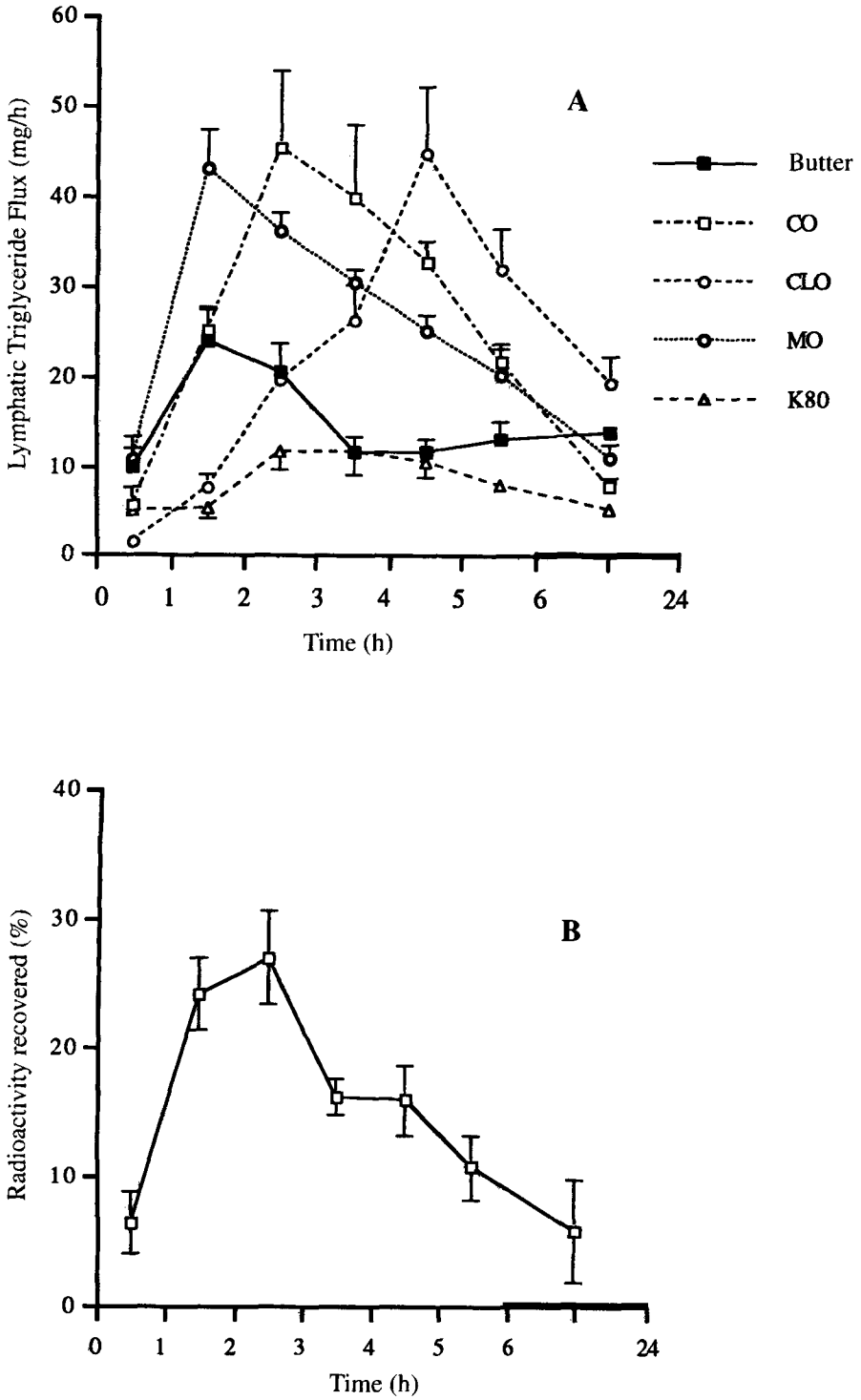


FIG. 1. A. Lymphatic triglyceride flux (mg/h) in rat mesenteric lymph following intragastric feeding. Values are means ± SEM (bars), $n = 6$, except for corn oil, $n = 3$. B. Time course recovery of radioactivity after intragastric feeding with butter and labeled oleic acid. Values are means ± SEM (bars), $n = 6$. Abbreviations: CO, corn oil; CLO, cod liver oil; MO, menhaden oil.

45.3 ± 8.6, and 44.7 ± 7.6 mg for MO, CO, and CLO, respectively). With butter, this value fell to 24.0 ± 3.3 mg, half as much as with the oils; for K80, the absorption peak was discrete and spread, with the highest triglyceride output one-fourth that of the other oils.

When labeled oleic acid was added to butter (Fig. 1B), of

the radioactivity recovered over the first 24 h, 75% was recovered over the first 6 h and 51% during the second and the third hours (24.1 ± 2.8 and 26.9 ± 3.6%) of intestinal absorption.

Time course appearance of some characteristic exogenous fatty acids in rat mesenteric lymph. An increase of the weight percentages of some characteristic fatty acids of the lipids ad-

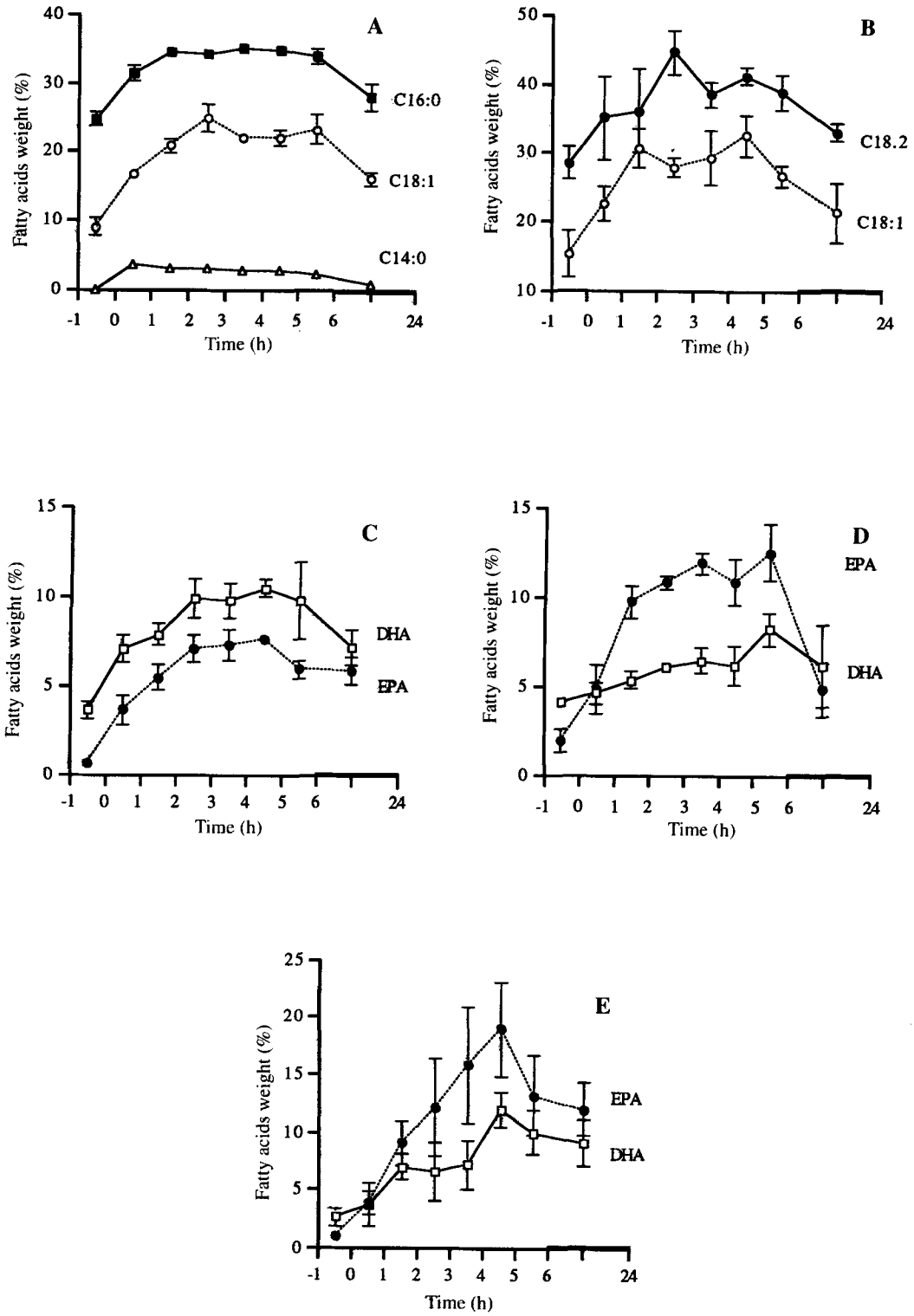


FIG. 2. Time course appearance of some characteristic fatty acids of butter, oils, or K80 in rat mesenteric lymph following intragastric feeding. Values are percentages of weight of fatty acids ± SEM (bars) $n = 4–6$; A, butter; B, corn oil; C, cod liver oil; D, menhaden oil; E, K80; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid..

ministered was observed as soon as the absorption process took place: oleic acid and butter feeding, oleic and linoleic acids and corn oil feeding, and particularly EPA and DHA and either the two marine oil feeding or EPA and DHA ethyl ester feeding. In fact, Figure 2 shows a more or less steady state of appearance in mesenteric lymph for these exogenous fatty acids, with comparison to the fasting state. For example, EPA and DHA were present in high percentages after the absorption peak and even beyond the first 6 h of absorption with the CLO and K80 experiments.

Usually, the fatty acid pattern of administered lipids was maintained in mesenteric lymph at the absorption peak. However, although the ratio of EPA to DHA was equal to 1.06 and 2.4 in CLO and MO, respectively, it was always lower in mesenteric lymph, even during the active absorption period. Thus, this ratio was equal to 0.7 and 1.8 with CLO and MO experiments, respectively, at the absorption peak. With K80 feeding, this ratio was sometimes larger or smaller than 1.6, the ratio of the mixture administered.

The weight percentage of the characteristic fatty acids recovered in mesenteric lymph was in parallel to the increased amounts of triglycerides measured in these same mesenteric lymph samples. However, the contribution of endogenous fatty acid to the formation of mesenteric lymph lipoprotein was also clearly apparent during exogenous lipid absorption processes.

Absorption of EPA and DHA appeared more efficient in marine oils than in the K80 experiments, taking into account their weight percentages in the two marine oils and ethyl esters K80 studied. The intestinal supply of EPA and DHA was higher in absolute value for the two marine oils compared with K80, with an average of 3.4–4.5 mg vs. 2.2 mg for EPA, and 4.3–4.5 mg vs. 1.5 mg for the DHA.

Mesenteric lymph lipoproteins. The absorption of butter, the three oils, or the ethyl esters of EPA and DHA administered to rats resulted in mesenteric lymph chylomicrons (Fig. 3). A correlation was noted between the amount of triglycerides recovered and the percentage and size of lipoproteins of diameter ≥0.1 μm. This correlation was particularly identified with CLO feeding ($r = 0.97$, $P < 0.005$). For this oil, chylomicrons were numerous but their size was often smaller than that observed with MO and CO feeding, in which the ab-

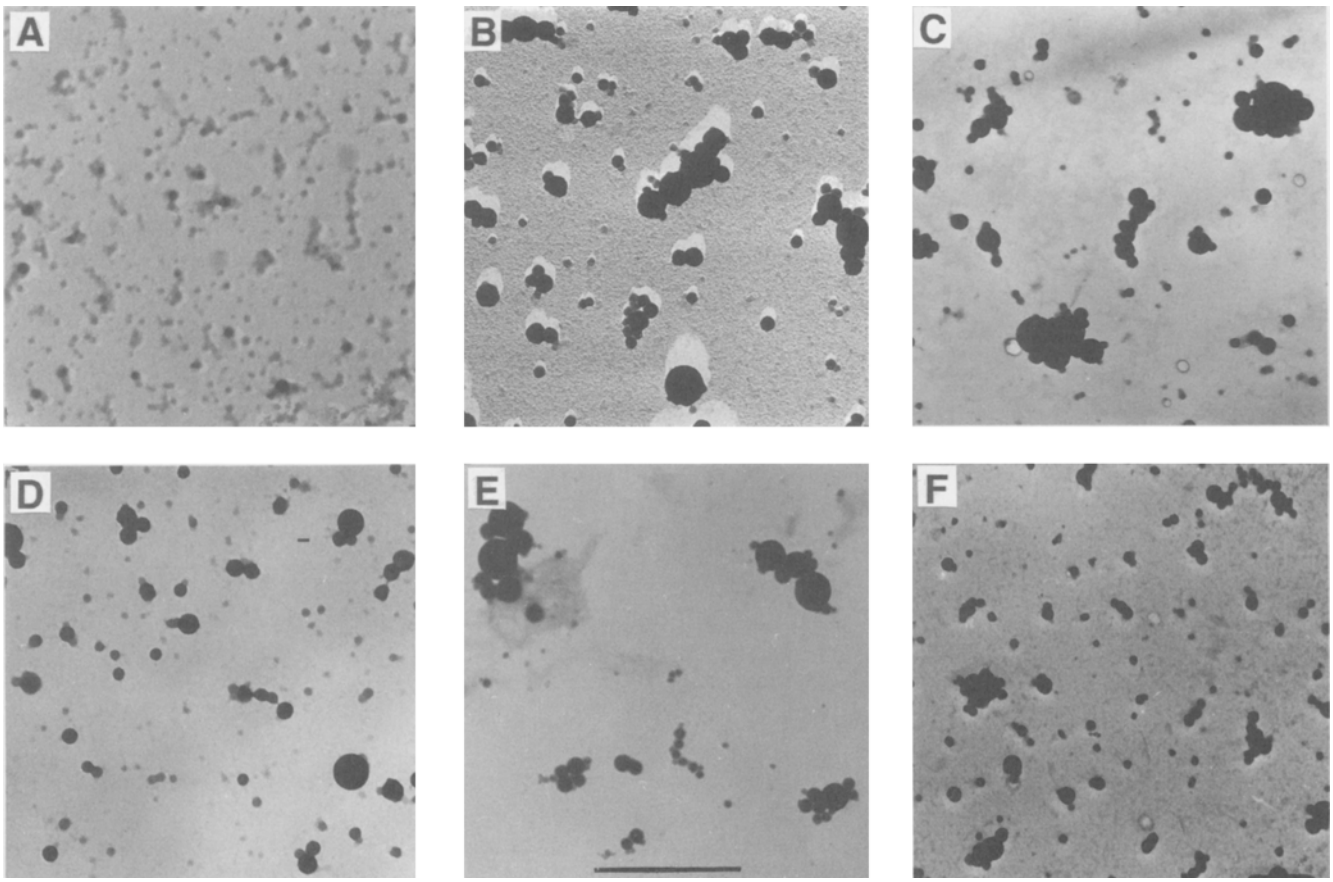


FIG. 3. Electron micrographs of lipoprotein particles of rat mesenteric lymph collected at the peak of absorption after intragastric administration of 0.65 mL of (B) butter, (C) 0.5 mL of corn oil, (D) cod liver oil, (E) menhaden oil, or (F) ethyl esters of eicosapentaenoic acid and docosahexaenoic (K80). Compared with mesenteric lymph of (A) fasting rats, absorption of lipids was followed by a scarce VLDL presence and a particular increase in enlarged chylomicrons especially after corn oil and menhaden oil absorption. Conversely, after absorption of K80, chylomicrons remained relatively small in agreement with biochemical results. Scale bar = 1 μm.

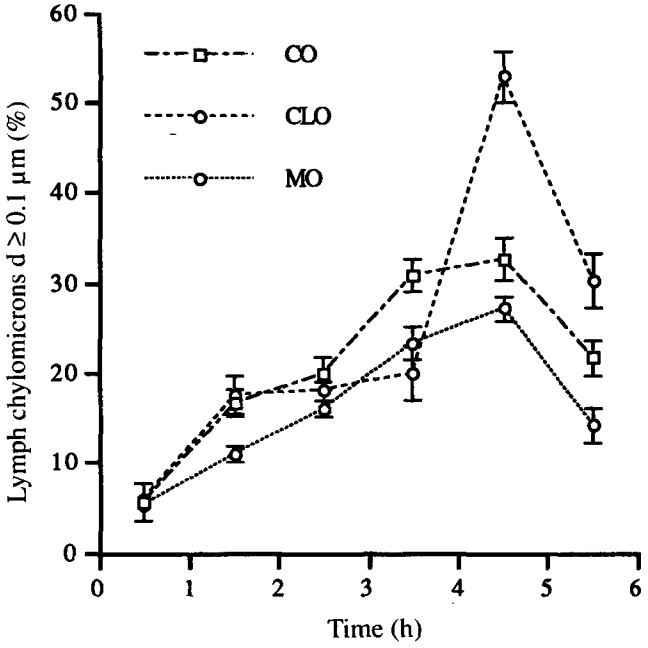


FIG. 4. Distribution of mesenteric lymph lipoproteins. Relative proportion of lipoproteins of a diameter ≥0.1 µm, in various time periods of mesenteric lymph collection after corn oil (CO), cod liver oil (CLO), or menhaden oil (MO) feeding (see the Materials and Methods section).Values are means ± SEM (bars).

sorption peaks occurred earlier (Fig. 1A). For these three oils, the amount of triglycerides carried by mesenteric lymph lipoproteins was equivalent at the absorption maximum and chylomicrons were observed with CO and MO. Consequently, the percentage of these large chylomicrons was higher with CLO feeding (Figs. 3 and 4). Whatever the calculation method used, all lymph particles or particles of a diameter larger than either 0.07 or 0.1 µm, the lymph particles after the feeding of ethyl ester of EPA and DHA were always smaller than after intragastric administration of butter or the three oils (Fig. 4).

DISCUSSION

In the fasting state, lymph flows of the rats were similar to those seen in previous studies carried out in comparable experimental conditions, and their rise during lipid absorption showed no significant difference regardless of the fat or oil given (17,24).

In agreement with Chernenko *et al.* (20), during the first 6 h or the following 18 h after either vegetable or marine oil feeding, there was no significant difference in lymph absorption. Because Chen *et al.* (29) added albumin to their lipid emulsion, which is known to decrease both fatty acid intestinal uptake and lymph flow (30), the discrepancy of our data with the observations of these authors might be explained by differences in experimental techniques. Indeed, intragastric administration, as used in the present study, seemed to allow lingual lipase hydrolysis, particularly at the *sn*-3 position of the triglycerides, and favored the start of lipid emulsification (6,31). Nelson and Ackman (2) emphasized the importance of the beginning of triglyceride hydrolysis in the stomach, and Harris and Williams (7), in human studies, demonstrated the very rapid and intensive absorption of emulsified fish oils.

Nelson and Ackman (2), believed that the rate of lipolysis could affect the route by which the liberated fatty acids enter the circulation. After butter feeding, the release of medium-chain fatty acids by lingual lipase activity during the stomach step, increased their portal absorption and explained the lower lymph triglyceride outputs in comparison with the oil experiments. Furthermore, this effect of the stomach on butter absorption could also explain differences with the results of Kalegoris and Story (22,32), who used intraduodenal butter administration. Such a practice delayed both emulsification and hydrolysis. Consequently, the absorption of fatty acid triglycerides was delayed, and endogenous fatty acid participation in lymph lipoproteins might have been increased (33).

In case of the oils, the faster the processes of absorption occurred during the first 6 h after feeding, the lower the amounts of lymph triglyceride required during the following 18 h of lymph collection. Such observations were to be linked with the involvement of endogenous fatty acid (33) which was maintained over a longer time period and, in the same way, might explain the high lymph triglyceride output obtained from the 7th to the 24th hour after CLO ingestion.

The slight increase in phospholipids in whole mesenteric lymphs observed with the two marine oils in comparison with the vegetable oil might be attributed to the n-3 fatty acids in these oils. This has been observed in previous studies, for example, between oleic acid and EPA (34–36).

EPA and DHA given as 80% of total fatty acids in ethyl esters were recovered in lymph samples at a level of 1% in accordance with previous reports (18,19,21,37,38); when given in the form of marine oils, peak values were 5–16%. This difference in absorption may reflect differences in emulsification, hydrolysis, and absorption. Previous reports have demonstrated in both rats and humans that ethyl esters are as well absorbed as triglycerides (18,19,21,38,39).

When n-3 polyunsaturated fatty acids were administered alone in ethyl ester form, in the absence of monoglycerides, their enterocyte esterification by the phosphatidic pathway was less efficient than with the monoglyceride pathway in accordance with the lower efficiency of lumenal hydrolysis (38,40). Indeed, Lawson and Hughes (19) showed that the absorption of fatty acid ethyl esters was highly dependent on the amount of co-ingested fat, a source of monoglycerides. As suggested by Ackman (12,13), it is necessary to point out that in the case of ethyl esters, transport in phospholipid form both by mesenteric lymph and portal blood is possible. This was confirmed by Hamasaki *et al.* (41), who observed a high incorporation of EPA administered as ethyl esters in plasma phospholipids and, in the present work, by the highest enrichment in phospholipids being in lymph after K80 feeding. In agreement with some observations by Yang and Kuksis (34) on CO and its fatty acid methyl esters, the absorption of EPA

and DHA in the form of ethyl esters *via* the glycerophosphate pathway released smaller lipoproteins in mesenteric lymph compared with those obtained with the three oils studied.

High percentages of short-chain (<16) fatty acids, particularly with butter and MO, were linked to an early appearance of triglycerides in mesenteric lymph. Conversely, the presence of very long-chain monounsaturated fatty acids in the CLO, such as gadoleic and cetoleic acids, delays and sometimes impairs the absorption of other fatty acids (21,28,42), thus delaying the lymph triglyceride absorption peak.

Taking into account the fatty acid distribution on the glycerol of the CO and MO triglycerides (21,43), the slight delay in the appearance of the absorption peak with CO might involve a more rapid hydrolysis of shorter-chain fatty acids of MO accompanied by a consecutive rapid enterocyte uptake of monoglycerides, rapidly available for triglyceride reesterification through the monoglyceride pathway. These results were in agreement with the high release of myristic, palmitoleic, and oleic acids observed by Yang *et al.* (38) studying MO lipolysis. Differences in mesenteric lymph triglyceride output profiles were in accordance with the size of mesenteric lymph lipoproteins. The size of the chylomicrons was dependent upon the amount of triglycerides absorbed and transported (44) and on the fatty acid composition of exogenous lipids (28,34). In our experimental conditions of lymph fixation and lipoprotein shadowing, distortions of the lipoprotein shapes were never observed. As previously shown (28), chylomicrons formed at the peak of absorption were larger than those formed at the beginning and toward the end of the absorption period. When absorption peaks were spread over a long period of time, a longer length of small intestine involved in absorption processes may explain an increase in chylomicon number. When the absorption peak was obtained over a shorter time period, for an equivalent triglyceride amount recovered in lymph, an increase in chylomicron size was observed (45). Such illustrations were consistent with the effect of the degree of unsaturation of triglyceride fatty acids. A certain degree of absorptive efficiency clearly appeared in a comparison of both the triglyceride output profiles and the morphological aspect of chylomicron formation and was confirmed especially after MO and CLO feeding, in agreement with previous studies (21,28,46) and as shown by Kalogeris and Story (22) after butter and CO feeding.

Aside from the consequences of the differential activity of preduodenal and pancreatic lipases according to the nature of fatty acid located at the *sn*-1 and *sn*-3 positions of triglycerides, fatty acid absorption is influenced by the triglyceride structure and is increased for fatty acids located in the *sn*-2 position, taking into account the preferential triglyceride synthesis *via* the 2-monoglyceride pathway (8,9,12,13,20,47).

Scrutiny of the time course appearance of some characteristic exogenous fatty acids illustrates these absorption modalities. The distribution of palmitic and oleic acids on the *sn*-1, *sn*-2, and *sn*-3 positions of the butter triglycerides was as follows: 31, 32.3, 5.4 for palmitic acid and 30.0, 18.9, 23.1 for oleic acid *sn* positions, respectively (48). Figure 1B shows the time course of mesenteric lymph appearance of butter containing labeled exogenous oleic acid, while in Figure 2, both endogenous and exogenous oleic acid were considered. Examination of the two profiles shows that oleic acid preferentially distributed in the *sn*-1 and *sn*-3 positions of butter triglycerides was consequently rapidly released and taken up by enterocytes and recovered in mesenteric lymph. Moreover, while the respective percentages (w/w) were 29 and 25% for the butter containing palmitic and oleic acids, palmitic acid absorption seemed to be favored by its preferential location in the *sn*-2 position.

Despite the preponderance of linoleic acid in the *sn*-2 position and the random distribution of oleic acid for the CO triglycerides (43), preferential lymph output of linoleic acid appeared only during the first 3 h of absorption.

Mesenteric lymph appearance profiles of n-3 polyunsaturated fatty acids in the present work were similar to those reported in the literature (20,49). Although great variation was seen, depending on the fish species and the area of fishing, DHA was often enriched at the *sn*-2 position of marine oil triglycerides, particularly for the CLO in which EPA is more randomly distributed. Thus, if the distribution of EPA and DHA on the *sn*-1, *sn*-2, *sn*-3 positions of CLO triglycerides was 2, 12, 13 and 1, 20, 6, respectively (11), the results obtained would be in accordance with the structure of marine oil triglyceride, and might explain that although there were percentages of 9 and 9.6% (w/w) of DHA and of EPA, respectively, in CLO, increased mesenteric absorption of DHA was observed after the feeding of this oil, in the same way as MO (38). Such results might provide evidence of a higher intestinal availability of DHA compared with EPA at the initial time period of absorption. Investigations are currently being carried out to attempt to link some of these intestinal events with the gastrocolic localization of acyltransferases and binding proteins involved in lipid intestinal absorption processes of lipids.

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Comparative Hypocholesterolemic Effects of Six Vegetable Oils in Cholesterol-Fed Rat

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ABSTRACT: The hypocholesterolemic efficacies of various polyunsaturated fatty acids were compared in rats given cholesterol-enriched diets. *Oenothera biennis* Linn oil (OBLO, linoleic + γ-linolenic), sunflower oil (linoleic), palm oil (PLO, oleic + linoleic), soybean oil (linoleic + α-linolenic), high-oleic safflower oil (oleic + linoleic), or mixed oil (linoleic + α-linolenic) was added to the diet at 200 g/kg (20% groups). OBLO was also added at 100 g/kg diet (10% group). The serum total and very low density lipoprotein + intermediate lipoprotein + low density lipoprotein cholesterol concentrations of the 10 and 20% OBLO groups were consistently lower than those in the other groups. The liver cholesterol concentration in the PLO group was lower in all groups. The liver cholesterol concentrations in the 10 and 20% OBLO groups were also lower than in the other groups. There were no significant differences in the fecal neutral sterol and bile acid extraction among groups.
Lipids 31, 415–419 (1996).

Dietary linoleic acid (LA, 18:2n-6) serves as a precursor for biosynthesis of arachidonic acid (20:4n-6), the substrate for eicosanoid synthesis through activity of the enzymes cyclooxygenase and 5-lipoxygenase. It has long been accepted as having hypocholesterolemic effects (1,2). More recently, linoleic acid derivatives, particularly γ-linolenic acid (GLA), were found to be even more potent in reducing blood cholesterol in humans and rats (3–6). Richard *et al.* (7) also reported that a dietary intake of 4–5.8 mmol GLA per day is more efficient in reducing serum total and low density lipoprotein (LDL) cholesterol levels than dietary LA in humans. However, natural sources of GLA are quite limited.

Evening primrose oil usually contains about 9% GLA. This oil has been shown to have greater hypocholesterolemic activity than safflower oil (SAFO) or soybean oil (SBO) at the 10% level in the diet (8,9). Suzuki *et al.* (10) have found that several genera of molds can produce lipids containing about 10% GLA. However, the mold oil contains less LA and more oleic and palmitic acids. Thus, the mold oil does not have greater hypocholesterolemic activity than SAFO (11). *Oenothera biennis* Linn seed oil (OBLO) (evening primrose) contains about 71% LA and 14% GLA. The purpose of the present study is to investigate the effect of OBLO on lipid metabolism in rats in comparison with several other plant oils.

MATERIALS AND METHODS

Animals and diets. Male F344/jcl rats (4 wk old) were purchased from Japan CLEA Co. Ltd. (Tokyo, Japan). Thirty-five animals were housed individually in cages in a room controlled for 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 or 20; cornstarch, 15; cellulose powder, 5; DL-methionine 0.3; mineral mixture, 3.5 (AIN-76) (12); vitamin mixture, 1 (AIN-76) (12); choline bitartrate, 0.2; cholesterol, 1; sodium cholate, 0.25; and sucrose to 100. OBLO was extracted from seeds collected in fields around the city of Obihiro (Hokkaido, Japan) using an oil mill (MKZA10-15; Masuko Sangyo Co., Ltd., Saitama, Japan). Sunflower oil (SFO) was a generous gift from the town of Hokuryu (Hokkaido, Japan). Palm oil (PLO), perilla oil, high-oleic SAFO, and SBO were purchased from Asahi Electric Industry Co. (Tokyo, Japan), Kanto Chemical Co. Inc. (Tokyo, Japan), Ajinomoto Co. (Tokyo, Japan), and Fuji Oil Co. (Osaka, Japan), respectively. Mixed oil (MIXO) was a mixture of PLO, high-oleic SAFO, and perilla oil (6:3:1, by vol) (13). The lipid class of each oil was as follows: OBLO was composed of 79% triacylglycerol (TG), 6% diacylglycerol (DG), 9% sterol, and other components; SFO was composed of 73% TG, 3% DG, 9% sterol, and other components; PLO was composed of 78% TG, 5% DG, 9% sterol, and other components; SAFO was composed of 84% TG, 2% DG, 9% sterol, and other components; SBO was composed of 80% TG, 1% DG, 13% sterol, and other components; MIXO was composed of 85% TG, 2% DG, 4% sterol, and other components. Sitosterol comprised about 81–91% of the sterol concentration in each oil.

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Abbreviations: DG, diacylglycerol; GLA, γ-linolenic acid; GLC, gas–liquid chromatography; HDL, high density lipoprotein; IDL, intermediate lipoprotein; LA, linoleic acid; LDL, low density lipoprotein; MIXO, mixed oil; OBLO, *Oenothera biennis* Linn oil; PC, phosphatidylcholine; PLO, palm oil; PUFA, polyunsaturated fatty acid; SAFO, safflower oil; SBO, soybean oil; SFO, sunflower oil; TG, triacylglycerol; VLDL, very low density lipoprotein.

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TABLE 1
Fatty Acid Composition of Dietary Fats

Dietary fats	Fatty acids (wt%)						
	16:0	18:0	18:1	18:2n-6	18:3n-3	18:3n-6	P/S[a]
Oenothera biennis							
Linn oil	7.2	1.4	6.2	71.0	—	13.7	11.7
Sunflower oil	6.6	3.6	25.1	63.5	—	—	9.6
Palm oil	48.8	0.4	33.9	16.9	—	—	0.4
Soybean oil	11.3	2.8	25.0	51.3	8.9	—	5.3
Safflower oil[b]	6.8	1.8	40.0	50.3	0.3	—	7.5
Mixed oil[c]	30.1	2.9	36.7	22.8	6.6	—	1.0

[a]Polyunsaturated/saturated ratio (P/S) = (18:2 + 18:3)/16:0.
[b]High-oleic safflower.
[c]The dietary fat was a mixture of palm oil, high-oleic safflower oil, and perilla oil (6:3:1, by vol).

Table 1 shows the fatty acid compositions of the dietary fats. All animals were fed a fat- and cholesterol-enriched diet containing 200 g of PLO and 10 g cholesterol/kg for 4 wk as a preliminary diet to create hypercholesterolemic rats. The rats (8 wk old) were divided into seven groups of five animals each. Rats were allowed free access to experimental diets and water for 6 wk; body weight and food consumption were recorded every other week. To avoid autooxidation, each diet was stored at −20°C and freshly prepared each day. All animal procedures described conformed to certain principles (see Ref. 14).

Analytical procedures. Blood samples (2 mL) were collected weekly between 0800 and 1000 h from the jugular veins of fed rats. The samples were taken into tubes without 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 6 wk, all fecal excretion during one day was collected; fecal dry weights did not differ among 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, high density lipoprotein (HDL)-cholesterol, and 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 very low density lipoprotein (VLDL) + intermediate lipoprotein (IDL) + LDL-cholesterol concentration was calculated as follows: VLDL + IDL + LDL-cholesterol = total cholesterol − HDL-cholesterol.

Total lipids were extracted from serum, liver, and feces by a mixture of chloroform/methanol (2:1, vol/vol) (15). The serum and liver phosphatidylcholine (PC) were separated by two-step single-dimensional thin-layer chromatography on a silica gel plate; the solvents were chloroform/methanol (one-step: 95:5, vol/vol) and chloroform/acetone/methanol/acetic acid/water (two-step: 50:20:15:10:5, by vol). The fatty acids of the PC fraction were esterified in HCl–methanol (5 mL/L) for 2 h at 125°C (16) and analyzed using gas–liquid chromatography (GLC; Shimadzu 14A, Kyoto, Japan) with a 10% DEGS packed column (2 mm × 2.6 m, support shimalite w; Shimadzu) with nitrogen as the carrier gas. Neutral sterols in feces and liver obtained by saponification were acetylated (17) and analyzed by GLC with a DB17 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA). Acidic sterols in feces were measured by GLC following the method of Grundy *et al.* (18).

Statistical analysis. Data are presented as means and standard deviations. The mean and standard deviation for serum total cholesterol, HDL-cholesterol, VLDL + IDL + LDL-cholesterol, and TG for each time point were calculated. The significance of differences between treatment groups was determined by general liner models 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. The dietary MIXO tended to increase the body weight gain significantly more than the other dietary fats except for dietary SBO. The liver weight in the 10% OBLO group was the lowest of all groups, and there were no significant differences in the food intake among the groups.

Tissue lipid concentration. The serum total, VLDL + IDL + LDL-cholesterol, HDL-cholesterol, and TG concentrations are shown in Table 3. The total and VLDL + IDL + LDL-cho-

TABLE 2
Body Weight Gain, Food Intake, and Liver Weight in Rats Fed Dietary Fats for Six Weeks[a]

Component	Diet						
	10% OBLO	20% OBLO	SFO	PLO	SBO	SAFO	MIXO
Body weight gain (g/6 wk)	97 ± 8[b]	98 ± 10[b]	99 ± 20[b]	94 ± 7[b]	107 ± 9[a,b]	93 ± 10[b]	116 ± 10[a]
Food intake (g/6 wk)	593 ± 51	591 ± 24	567 ± 68	601 ± 24	573 ± 55	599 ± 54	609 ± 40
Liver weight (dry g/100 g body weight)	2.6 ± 0.2[b]	3.5 ± 0.2[a]	3.4 ± 0.4[a]	3.4 ± 0.3[a]	3.4 ± 0.1[a]	3.3 ± 0.3[a]	3.4 ± 0.2[a]

[a]Values are expressed as means ± standard deviations for five rats. Means within the same rows bearing different superscripts are significantly different ($P < 0.05$). OBLO, *Oenothera biennis* Linn oil; SFO, sunflower oil; PLO, palm oil; SBO, soybean oil; SAFO, safflower oil; MIXO, mixed oil.

TABLE 3
Serum Total Cholesterol, VLDL + IDL + LDL-Cholesterol, HDL-Cholesterol, and Triacylglycerol Concentrations in Rats Fed Dietary Fats for Six Weeks[a]

Diet	0 Wk	3 Wk	6 Wk
Total cholesterol (mmol/L)			
10% OBLO	12.1 ± 3.0	4.4 ± 0.7^{c}	3.5 ± 0.4^{c}
20% OBLO	15.1 ± 2.7	4.2 ± 0.5^{d}	3.7 ± 0.6^{c}
SFO	14.8 ± 1.8	7.7 ± 0.6^{b}	6.2 ± 1.1^{b}
PLO	14.0 ± 1.2	9.5 ± 1.1^{a}	9.3 ± 2.1^{a}
SBO	14.5 ± 2.5	6.6 ± 0.9^{b}	5.1 ± 0.6^{b,c}
SAFO	13.2 ± 2.7	8.1 ± 2.1^{a,b}	6.9 ± 2.2^{b}
MIXO	15.7 ± 3.0	6.9 ± 1.9^{b}	6.1 ± 0.7^{b}
VLDL + IDL + LDL-cholesterol (mmol/L)			
10% OBLO	11.0 ± 2.9	2.9 ± 0.6^{c}	2.1 ± 0.2^{c}
20% OBLO	13.7 ± 2.5	3.2 ± 0.3^{c}	2.4 ± 0.7^{c}
SFO	13.4 ± 1.9	6.9 ± 0.7^{a,b}	5.3 ± 1.2^{b}
PLO	12.6 ± 1.3	7.9 ± 1.0^{a}	7.2 ± 1.8^{a}
SBO	13.1 ± 2.4	5.7 ± 0.9^{b}	4.3 ± 0.7^{b}
SAFO	11.9 ± 2.6	7.2 ± 2.1^{a,b}	5.6 ± 1.8^{b}
MIXO	14.5 ± 3.0	5.9 ± 1.9^{b}	4.9 ± 0.7^{b}
HDL-cholesterol (mmol/L)			
10% OBLO	1.1 ± 0.1^{b}	1.5 ± 0.2^{a}	1.4 ± 0.3^{b}
20% OBLO	1.5 ± 0.3^{a}	1.1 ± 0.1^{b}	1.4 ± 0.3^{b}
SFO	1.4 ± 0.1^{a,b}	0.8 ± 0.2^{c}	0.9 ± 0.2^{b}
PLO	1.4 ± 0.2^{a,b}	1.5 ± 0.3^{a}	2.1 ± 0.3^{a}
SBO	1.4 ± 0.1^{a,b}	0.9 ± 0.1^{b,c}	0.9 ± 0.2^{b}
SAFO	1.4 ± 0.2^{a,b}	0.9 ± 0.0^{b,c}	1.3 ± 0.7^{b}
MIXO	1.2 ± 0.2^{a,b}	1.0 ± 0.1^{b,c}	1.2 ± 0.1^{b}
Triacylglycerol (mmol/L)			
10% OBLO	0.8 ± 0.3	1.5 ± 0.6^{a}	1.3 ± 0.3^{a}
20% OBLO	1.2 ± 0.5	0.7 ± 0.3^{b,c}	0.6 ± 0.3^{b}
SFO	1.1 ± 0.6	0.4 ± 0.3^{c}	0.5 ± 0.1^{b}
PLO	1.0 ± 0.5	1.2 ± 0.5^{a,b}	1.5 ± 0.8^{a}
SBO	1.0 ± 0.5	0.5 ± 0.3^{c}	0.5 ± 0.2^{b}
SAFO	0.7 ± 0.3	0.6 ± 0.4^{c}	0.6 ± 0.3^{b}
MIXO	0.9 ± 0.2	0.9 ± 0.5^{b,c}	0.6 ± 0.2^{b}

[a]Values are expressed as means ± standard deviations for five rats. Means within the same columns bearing different superscripts are significantly different ($P < 0.05$); VLDL, very low density lipoprotein; IDL, intermediate lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein. See Table 2 for other abbreviations.

lesterol concentrations in the 10 and 20% OBLO groups were significantly lower than those in the other groups. Although HDL-cholesterol concentrations in the 10 and 20% OBLO groups were significantly lower than those in the PLO group, there were no significant differences among all groups except for dietary PLO. The serum TG concentrations in the 10% OBLO and PLO groups were significantly higher than those in the other groups at 6 wk.

Table 4 illustrates the cholesterol concentrations in the livers of rats at the end of the 6-wk feeding period. Dietary SFO increased liver cholesterol concentration significantly among all groups, whereas dietary PLO and 10% OBLO groups showed a significant decrease. The dietary 20% OBLO, SAFO, and MIXO groups also showed lower liver cholesterol concentration than the SFO and SBO groups.

Fatty acid composition of the tissue. The fatty acid composition of PC in the serum and liver is shown in Table 5. The level of 18:2n-6 in the serum of the 20% OBLO group was significantly higher than that in the PLO, SBO, SAFO, and MIXO groups. The level of 20:3n-6 in the 10% OBLO group was the highest among all groups. The level of 20:4n-6 showed a greater increase in the serum of the SFO groups than that in the SBO and MIXO groups.

The level of 18:2n-6 in the liver of the 20% OBLO and SBO groups increased significantly compared with the PLO and MIXO groups. The level of 20:3n-6 in the 10 and 20% OBLO groups was significantly higher than in the other groups.

Fecal lipid concentration. Table 6 shows the effects of the dietary fat on fecal neutral sterol and bile acid concentrations in rats at the end of the experimental period. There were no significant differences in the fecal neutral sterol concentrations among the groups.

The excretion of lithocholic acid increased in the PLO group, and the excretion of cholic acid decreased in the SAFO and MIXO groups. However, there were no significant differences in the fecal total bile acid concentrations among the groups.

DISCUSSION

This study indicated that dietary OBLO at a level of ~190 mg 18:3n-6/d is more efficient in lowering serum total cholesterol than are SFO or SAFO which contains abundant LA, or MIXO with n-6/n-3 ratios of approximately 3.5 and a polyunsaturated/saturated ratio of approximately 1.0 (13). These results agree with those of previous studies (3–9). There were no significant differences in the fecal neutral sterol and total bile acid concentrations among all groups. These results show that the cholesterol-lowering effect of their polyunsaturated oils is not due to increased fecal excretion of neutral and acidic sterols. Huang *et al.* (8) reported that evening primrose oil (Efamol) lowered the plasma cholesterol concentration and increased the levels of LA and arachidonic acid in phospholipids of

TABLE 4
Liver Cholesterol Concentration in Rats Fed Dietary Fats for Six Weeks[a]

	Diet						
Component	10% OBLO	20% OBLO	SFO	PLO	SBO	SAFO	MIXO
Cholesterol (μmol/g wet liver)	48 ± 7^{d}	65 ± 15^{c}	109 ± 21^{a}	39 ± 5^{d}	91 ± 19^{b}	66 ± 17^{c}	67 ± 14^{c}

[a]Values are expressed as means ± standard deviations for five rats. Means within the same row bearing different superscripts are significantly different ($P < 0.05$). See Table 2 for abbreviations.

TABLE 5
Fatty Acid Composition of Phosphatidylcholine in Serum and Liver of Rats Fed Dietary Fats for Six Weeks[a]

Fatty acid	Diet						
	10% OBLO	20% OBLO	SFO	PLO	SBO	SAFO	MIXO
	(wt%)						
Serum phosphatidylcholine							
16:0	24.3 ± 1.6[b]	24.2 ± 2.8[b]	18.1 ± 2.3[c]	23.7 ± 1.5[b]	24.4 ± 2.0[b]	22.8 ± 3.3[b]	29.9 ± 7.8[a]
16:1n-7	0.9 ± 0.2[a,b]	0.6 ± 0.2[b]	1.0 ± 0.2[a,b]	1.4 ± 0.1[a]	0.8 ± 0.2[a,b]	1.4 ± 0.9[a]	1.0 ± 0.4[a,b]
18:0	20.2 ± 2.7[a,b]	17.0 ± 3.6[b]	23.3 ± 0.7[a]	19.7 ± 1.8[a,b]	22.7 ± 4.4[a]	20.0 ± 2.8[a,b]	20.5 ± 3.9[a,b]
18:1n-9	7.9 ± 0.7[c]	7.4 ± 1.9[c]	10.2 ± 1.0[c]	22.9 ± 2.7[a]	13.1 ± 3.1[b,c]	20.7 ± 7.4[a]	17.0 ± 6.6[a,b]
18:2n-6	30.9 ± 3.0[a,b]	33.6 ± 3.1[a]	30.9 ± 2.9[a,b]	21.4 ± 2.8[d]	27.3 ± 3.1[b,c]	23.6 ± 3.8[c,d]	19.7 ± 7.1[d]
18:3n-6	0.9 ± 0.6	0.5 ± 0.2	<0.1	<0.1	<0.1	<0.1	<0.1
18:3n-3	<0.1	<0.1	<0.1	<0.1	0.6 ± 0.2	<0.1	0.7 ± 0.3
20:3n-6	3.7 ± 0.7[a]	2.2 ± 0.7[b]	1.4 ± 0.3[c,d]	1.9 ± 0.3[b,c]	1.4 ± 0.4[c,d]	0.8 ± 0.4[d]	1.5 ± 0.7[b,c]
20:4n-6	11.2 ± 2.1[a,b,c]	14.5 ± 2.1[a,b]	15.1 ± 1.8[a]	9.4 ± 1.7[c]	9.7 ± 2.0[b,c]	10.8 ± 4.4[a,b,c]	9.7 ± 6.6[b,c]
Liver phosphatidylcholine							
16:0	25.4 ± 6.6	22.6 ± 3.7	25.8 ± 6.0	26.0 ± 1.8	21.4 ± 1.9	22.6 ± 3.0	27.0 ± 4.2
16:1n-7	2.0 ± 1.7	2.4 ± 0.8	2.8 ± 2.3	2.1 ± 1.1	2.1 ± 1.3	1.6 ± 1.4	2.6 ± 2.0
18:0	17.0 ± 5.2	16.0 ± 2.2	20.9 ± 8.3	18.2 ± 2.4	17.8 ± 4.7	20.9 ± 3.9	19.9 ± 6.4
18:1n-9	22.3 ± 12.5	17.5 ± 10.5	21.1 ± 5.7	28.5 ± 5.6	19.3 ± 8.3	21.1 ± 6.7	24.2 ± 13.5
18:2n-6	14.1 ± 5.4[a,b]	17.0 ± 3.9[a]	13.4 ± 5.8[a,b]	9.2 ± 1.2[b]	18.4 ± 4.3[a]	14.3 ± 2.8[a,b]	10.3 ± 3.9[b]
18:3n-6	0.8 ± 0.4	1.0 ± 0.6	<0.1	<0.1	<0.1	<0.1	<0.1
18:3n-3	<0.1	<0.1	<0.1	<0.1	1.1 ± 0.5	<0.1	1.2 ± 1.2
20:3n-6	2.5 ± 1.9[a]	2.2 ± 0.5[a]	0.7 ± 0.3[b]	0.9 ± 0.2[b]	1.1 ± 0.5[b]	0.7 ± 0.2[b]	1.1 ± 0.7[b]
20:4n-6	16.0 ± 11.0	21.4 ± 9.7	15.2 ± 5.9	15.1 ± 5.0	18.9 ± 5.2	18.8 ± 5.6	13.7 ± 7.2

[a]Values are expressed as means ± standard deviations for five rats. Means within the same rows bearing different superscripts are significantly different ($P < 0.05$). See Table 2 for abbreviations.

plasma and liver more than hydrogenated coconut oil and SAFO. However, there were no significant differences in the liver cholesterol concentration among the three oils (8). Their results were similar to our data. However, the liver cholesterol concentrations in the groups fed diets containing more polyunsaturated fatty acids (PUFA) were higher than those in rats fed the dietary PLO. The low level of liver cholesterol in the PLO group may be attributable to the reduced hepatic LDL receptor activity, due to feeding fats containing less PUFA and more saturated fatty acid (19).

The reduction of the VLDL + IDL + LDL-cholesterol concentration was similar to that reported by Richard *et al.* (7). Ishikawa *et al.* (20) observed significant changes in plasma lipids after administration of 300 mg/d of GLA to hypercholesterolemic patients over an 8-wk period. The total cholesterol, LDL-cholesterol, and apo B concentrations were decreased in patients without hypertriglyceridemia. The effects on blood cholesterol therefore appeared to be clearly related to the dose of GLA administered, as judged by feeding 200–400 mg of GLA in this experiment. The high levels of serum total, HDL-, and VLDL + IDL + LDL-cholesterol in the PLO group were similar to those reported by Nicolosi *et al.* (21). They indicated that the high degree of dietary fatty acid saturation such as that found in coconut oil induced greater changes in plasma lipoprotein (apo B and apo A-I levels) and LDL metabolism (21). Dietary PLO increased the HDL-concentration among all groups. Sugano *et al.* (9) reported that olive oil containing less PUFA and more saturated

TABLE 6
Fecal Steroid Concentration in Rats Fed Dietary Fats for Six Weeks[a]

Component	Diet						
	10% OBLO	20% OBLO	SFO	PLO	SBO	SAFO	MIXO
	(μmol/100 g body weight per day)						
Cholesterol	38.1 ± 13.6	24.3 ± 4.2	39.4 ± 15.9	34.5 ± 20.5	24.0 ± 18.9	34.7 ± 17.4	22.4 ± 10.3
Coprostanol	1.15 ± 0.68	1.09 ± 0.43	1.67 ± 0.70	0.65 ± 0.91	0.69 ± 0.72	0.60 ± 0.43	3.21 ± 6.13
LCA	0.43 ± 0.19[a,b]	0.22 ± 0.10[b]	0.35 ± 0.16[a,b]	1.05 ± 1.62[a]	0.26 ± 0.23[a,b]	0.14 ± 0.15[b]	0.21 ± 0.24[b]
DCA	3.15 ± 2.05	3.19 ± 1.68	4.90 ± 2.73	3.33 ± 2.14	3.69 ± 3.10	2.00 ± 2.26	2.22 ± 2.09
CDCA	0.45 ± 0.86	0.09 ± 0.04	0.05 ± 0.04	0.23 ± 0.35	0.12 ± 0.09	0.03 ± 0.03	0.04 ± 0.04
CA	0.26 ± 0.16[a,b]	0.45 ± 0.36[a]	0.21 ± 0.23[a,b]	0.22 ± 0.18[a,b]	0.31 ± 0.39[a,b]	0.07 ± 0.08[b]	0.08 ± 0.08[b]
TBA	4.28 ± 3.13	3.96 ± 1.68	5.52 ± 3.10	4.83 ± 3.65	4.38 ± 3.70	2.25 ± 2.52	2.55 ± 2.43

[a]Values are expressed as means ± standard deviations for five rats. Means within the same rows bearing different superscripts 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.

fatty acid reduces the HDL-cholesterol concentration. Grundy and Denke (22) reported that LA lowers the level of apo A-I, which is the major protein component of circulating HDL (23).

In conclusion, the results of this study demonstrate that the OBLO decreases the serum total cholesterol and VLDL + IDL + LDL-cholesterol concentrations in the presence of excess cholesterol in the diet.

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A Rapid Method for Separation of Plasma Low and High Density Lipoproteins for Tocopherol and Carotenoid Analyses

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ABSTRACT: Ultracentrifugation (UC) is the method most often employed for separation and quantification of lipoproteins. Because this procedure requires expensive laboratory equipment, a large volume of fresh sample and an inordinate amount of time, it may not be ideal for routine clinical/experimental use. The aim of the current study was to evaluate a method which combines selective precipitation (HDL–P) and immunoseparation (LDL–I) for the rapid and reliable isolation of high density lipoproteins (HDL) and low density lipoproteins (LDL) specifically for vitamin E and carotenoid determination within these fractions. Cholesterol and triacylglycerol concentrations within the HDL and LDL were also determined to enable expression of vitamin E and carotenoid concentrations per gram of lipid. Isolation of lipoproteins by UC was used as the reference method (HDL–UC/LDL–UC). There were no significant differences between methods for α- and γ-tocopherol in LDL and HDL. Carotenoids measured in HDL and LDL were comparable between the methods. The exception was higher lutein/zeaxanthin concentration in HDL–P and LDL–I compared to HDL–UC and LDL–UC, respectively. Additionally, lycopene concentration was significantly lower in LDL–I compared to LDL–UC. In comparing vitamin E and carotenoid values in lipoproteins separated from fresh and frozen plasma by the direct method, there was no difference in α-tocopherol or the majority of carotenoids measured. In conclusion, a combination of selective precipitation and immunoseparation of fresh or frozen plasma for subsequent α- and γ-tocopherol analyses provides an accurate and reliable alternative to lipoprotein separation by UC. Additionally, carotenoid concentrations in HDL separated by selective precipitation and analyses of α- and β-carotenes and β-cryptoxanthin in LDL separated by immunoseparation are also reliable, while lycopene and lutein/zeaxanthin concentrations in LDL–I are not readily comparable to LDL–UC.

Lipids 31, 421–426 (1996).

Studies of the metabolism and transport of lipids and lipid-soluble nutrients rely on the ability to separate and quantify plasma lipoproteins. Further, an accurate and precise measurement of lipoprotein constituents is dependent on the separation technique employed. Ultracentrifugation (UC) is the technique traditionally used for separation and quantification of lipoproteins on the basis of hydrated density (1). This method requires expensive equipment and significant sample volume. It is also time-consuming, requiring several days. Additionally, fresh plasma and serum must be utilized for the separation to obtain optimal results. These aspects of the UC methodology clearly limit its utility for routine laboratory analyses.

Alternative methods have been developed for lipoprotein separation. Column chromatography, high-performance liquid chromatography (HPLC), and electrophoresis are not applicable for routine analyses as they are time-consuming, and accuracy is not always satisfactory (1). For the routine separation and subsequent cholesterol determination of high density lipoproteins (HDL), selective precipitation of apolipoprotein B containing particles is now widely used in routine analyses (2). This method has the advantages of being fast, reliable, and inexpensive. Chemical precipitation and affinity chromatography have been proposed and used for the isolation of low density lipoproteins (LDL) (3,4). While these methods are also inexpensive and efficient, LDL cannot be separated from very low density lipoproteins (VLDL) and chylomicrons. Also, only fasting blood samples and samples with low to normal triacylglycerol (TG) concentrations can be separated with accuracy. Recently a new method was developed by Genzyme Corporation, Diagnostics Division (Cambridge, MA) and is now commercially available from Sigma Diagnostics (St. Louis, MO). That methodology permits the direct separation of LDL particles from other lipoproteins by immunoseparation. Chylomicrons, VLDL, and HDL are selectively removed and retained on a filter by specific antibodies immobilized on polystyrene microspheres that allow only LDL to pass through the filter. In a recent study that compared immunoseparation against ultracentrifugation in 115 serum samples, McNamara *et al.* (5) concluded that immunoseparation is an accurate and reliable method for LDL cholesterol measurement.

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Abbreviations: HDL, high density lipoproteins; HDL–P, HDL isolated by selective precipitation; HDL–UC, HDL isolated by sequential ultracentrifugation; HPLC, high-performance liquid chromatography; LDL, low density lipoprotein; LDL–I, LDL isolated by immunoseparation; LDL–UC, LDL isolated by sequential ultracentrifugation; TG, triacylglycerol; UC, ultracentrifugation; VLDL, very low density lipoproteins.

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The objective of this study was to evaluate a method which permits fast and reliable isolation of HDL and LDL for subsequent accurate analyses of tocopherols and carotenoids in these lipoproteins. For this purpose, analyses of carotenoids and α- and γ-tocopherols in LDL separated by immunoseparation (LDL–I) and HDL isolated by selective precipitation (HDL–P) (herein referred to as direct methods) were compared to those in lipoproteins isolated by sequential UC (LDL–UC and HDL–UC). In addition, the effect of sample freezing (–80°C) on carotenoid and tocopherol concentrations following lipoprotein separation by LDL–I and HDL–P assays was assessed.

MATERIALS AND METHODS

Subjects. Twenty healthy, normolipidemic volunteers, eleven men and nine women, participated in the study. Fasting blood samples were drawn from each subject into vacutainers containing EDTA (0.15%). Plasma was separated by low-speed centrifugation for 20 min at 1,500 × *g* at 4°C. Lipoprotein separation procedures were started within an hour after blood sampling. Figure 1 summarizes the protocol for lipoprotein separation and vitamin analyses. All subjects received a description of the study protocol and signed informed consent forms. This study was approved by the University of Connecticut Human Subject Approval Committee.

Ultracentrifugation. The lipoprotein fractions (VLDL, LDL, and HDL) were isolated using two sequential ultracentrifugation steps. In brief, plasma (5 mL) was transferred to quickseal tubes (Beckman Instruments, Palo Alto, CA) and centrifuged for 18 h at 39,000 rpm, 4°C in a 40.3 Ti fixed-angle rotor ultracentrifuge (Beckman). The 1.006 g/mL top fraction (VLDL) was brought back to a volume of 2.5 mL with saline (0.85%). The bottom fraction was adjusted to a relative density of 1.063 with KBr and centrifuged for 18 h at 39,000 rpm to obtain the LDL (top) and the HDL (bottom) fractions. The HDL fraction was not further separated from the lipoprotein free fraction. Preliminary studies demonstrated that no carotenoids and tocopherols were associated with this fraction (Vogel, S., Contois, J.H., and Lammi-Keefe, C.J., unpublished observation).

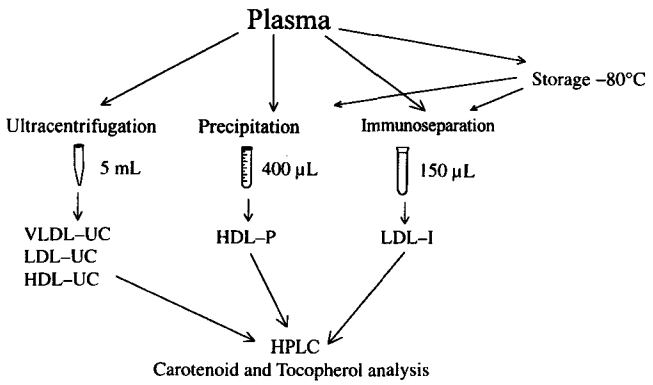


FIG. 1. Protocol for lipoprotein separation and vitamin analyses; HPLC, high-performance liquid chromatography; VLDL–UC, very low density lipoprotein isolated by sequential ultracentrifugation; LDL–UC, low density lipoprotein isolated by sequential ultracentrifugation; HDL–UC, high density lipoprotein isolated by sequential ultracentrifugation; HDL–P, high density lipoprotein isolated by selective precipitation; LDL–I, low density lipoprotein isolated by immunoseparation.

Immunoseparation. A commercially available reagent (Genzyme Corporation) was used to separate LDL from plasma by immunoseparation. This method uses specific antibodies immobilized on polystyrene microspheres to selectively remove HDL, chylomicrons, and VLDL, which are excluded by a filter. LDL passes through the filter and is present in the filtrate. For our purpose, we first pipetted 334 µL of the reagent into separation tubes (Genzyme Corporation), added 50 µL plasma, vortexed the tubes immediately, and allowed them to incubate for 10 min at room temperature. LDL particles were drawn through the filter with subsequent centrifugation at full speed in a microfuge at 12,000 × *g* for 10 minutes. Because of the approximate sevenfold dilution of the LDL, three filtrates for each sample were pooled to ensure adequate sample size for the detection of tocopherols and carotenoids in the LDL fraction.

Selective precipitation. HDL were isolated from plasma according to Warnick *et al.* (6), using Dextran Sulfate-Mg^{2+} chloride as the precipitation reagent. Plasma (400 µL) was transferred to microtubes, and 40 µL reagent added to each tube. The tubes were immediately vortexed. After a 10-min incubation at room temperature, the samples were centrifuged for 30 min at 1,500 × *g*. The supernate containing the HDL particles was then removed and kept at 4°C until further analyses.

Lipid determination. Cholesterol and TG concentrations in plasma and lipoproteins were measured using enzymatic test kits (Sigma Diagnostics). Lipids in HDL–P were determined in 12 subjects.

Vitamin analyses. The α- and γ-tocopherols and carotenoids in plasma and lipoprotein fractions were analyzed using the reversed-phase HPLC method described by Barua *et al.* (7). Prior to extraction, retinyl hexanoate and β-apo-8′-carotenyl myristate in methanol were added to each sample as internal standards for tocopherols and carotenoids, respectively. After precipitation of proteins with methanol, all samples were extracted twice with hexane. No additional extraction with ethylacetate was performed. Preliminary analyses showed recovery was not improved with the ethylacetate extraction (Contois, J.H., and Lammi-Keefe, C.J., unpublished observation). The hexane extracts were transferred to test tubes and the hexane subsequently evaporated under a gentle stream of nitrogen. The residues were redissolved in 60 µL of a dichloromethane/mobile phase (1:5) mixture, and 40 µL were used for HPLC analyses. The mobile phase consisted of acetonitrile/methylenechloride/methanol/*n*-butanol with 0.1% ammonium acetate (90:15:10:0.1,by vol). The HPLC system included a Waters pump (flow rate 1.5 mL/min), a 5 µm C_{18} stainless-steel Waters (Milford, MA) resolve column, an autosampler (Millipore, Milford, MA), two absorbance detectors connected in series, and a dual channel integrator. The detectors were set at 300

TABLE 1
Carotenoid and Tocopherol Concentrations in Lipoproteins Used for Precision Determinations (μmol/L)[a,b]

Vitamin	HDL–P[c]	LDL–I[c]
Lutein/zeaxanthin	0.23 ± 0.03	0.21 ± 0.02
β-Cryptoxanthin	0.03 ± 0.003	0.03 ± 0.01
Lycopene	0.07 ± 0.01	0.22 ± 0.08
α-Carotene	0.02 ± 0.003	0.03 ± 0.01
β-Carotene	0.03 ± 0.002	0.10 ± 0.03
α-Tocopherol	7.76 ± 1.07	7.62 ± 0.68
γ-Tocopherol	2.37 ± 0.41	1.21 ± 0.11

[a]All values expressed as mean ± SD.
[b]Precision for analyses of lipoprotein isolated by direct methods only.
[c]Low density lipoprotein isolated by immunoseparation (LDL–I) and high density lipoprotein isolated by selective precipitation (HDL–P) refer to separation by immunoseparation and precipitation, respectively.

nm (Thermo Separation Products, Fremont, CA) to monitor retinol and tocopherols and at 450 nm (Varian 2550; Varian, Palo Alto, CA) to monitor carotenoids. The analyte peaks were identified by retention times and quantified using standard curves of external standards for each analyte.

Analyses of frozen samples. Samples of plasma from 12 subjects were stored for 6 mon at −80°C. After thawing of samples at room temperature, the LDL fraction was separated by immunoseparation and HDL by selective precipitation, as described above. Cholesterol, TG, and vitamin concentrations were determined for plasma and all lipoprotein fractions.

Statistical analyses. Concentrations of lipids (TG and cholesterol) and vitamins in the lipoprotein fractions separated by UC vs. the direct methods and concentration in fresh vs. frozen/thawed specimens were compared using paired *t*-tests (SAS Institute, Cary, NC).

RESULTS

Precision. To evaluate the applicability of the direct methods for the analyses of carotenoids and tocopherols, the variability was determined for the analyses of carotenoids and tocopherols in LDL and HDL isolated using the direct methods. Precision exceeding 95% was achieved for duplicate analyses of all HDL carotenoids and tocopherols. Precision for α- and γ-tocopherols in LDL was 99% and for carotenoids 86–97%. Table 1 shows lipoprotein concentrations of the carotenoids and tocopherols in the lipoproteins that were analyzed.

During separation, the LDL fraction was diluted approximately sevenfold, resulting in low carotenoid concentrations. Low carotenoid concentrations resulted in high variability associated with their detection by HPLC. We found that the use of LDL–I isolated from 300 μL plasma instead of 150 μL plasma decreased variability and increased precision to 95% for all carotenoids in the LDL fraction.

Comparison of direct methods and ultracentrifugation for separating LDL and HDL: TG and cholesterol. The mean plasma TG and cholesterol concentrations for 20 subjects were 86.6 ± 39.4 and 173.4 ± 33.4 mg/dL, respectively. There were no significant differences between the methods for LDL. The mean LDL cholesterol concentrations were 119.1 ± 45.1 and 110.3 ± 35.7 mg/dL, and the mean LDL–TG concentrations were 36.6 ± 40.8 and 24.3 ± 15.0 mg/dL for LDL–I and LDL–UC, respectively. In contrast, HDL lipid concentrations in 12 subjects were significantly different between the methods. HDL–TG concentrations (18.2 ± 6.4 vs. 13.4 ± 2.5 mg/dL, HDL–P vs. HDL–UC) and HDL cholesterol concentrations (51.1 ± 15.8 vs. 48.3 ± 13.9 mg/dL, HDL–P vs. HDL–UC) were significantly higher in HDL–P compared to HDL–UC.

Carotenoids and tocopherols. Mean carotenoid and tocopherol concentrations for LDL and HDL isolated from both separation methods are shown in Table 2. All values are expressed as μmol/g total lipid measured for the corresponding lipoprotein.

There were no significant differences between methods for α- and γ-tocopherol concentrations within either LDL or HDL. In LDL fractions, there were no differences for α-carotene, β-carotene, and β-cryptoxanthin concentrations between separation methods. In contrast, lycopene concentrations determined in LDL–I were significantly lower than those measured in LDL–UC, while lutein/zeaxanthin concentrations were significantly higher in LDL–I. There was very good comparability between methods for the majority of the carotenoids in the HDL fraction. Lutein/zeaxanthin was the exception with a significantly higher concentration in HDL–P.

Effect of storage on separation of LDL and HDL by the direct methods. In addition to comparing two lipoprotein separation methods, this study also investigated whether selective precipitation and immunoseparation could be applied to samples that had been previously frozen (−80°C) and then thawed.

TABLE 2
Carotenoid and α- and γ-Tocopherol Concentrations in LDL–I and HDL–P vs. LDL–UC and HDL–UC (μmol/g total lipid)[a]

Vitamin	N	LDL–I	LDL–UC	*P* value	HDL–P	HDL–UC	*P* value
Lutein/zeaxanthin	20	0.20 ± 0.12	0.14 ± 0.07	$P < 0.05$	0.37 ± 0.18	0.30 ± 0.11	$P < 0.05$
β-Cryptoxanthin	20	0.08 ± 0.07	0.10 ± 0.08	N.S.	0.12 ± 0.09	0.11 ± 0.08	N.S.
Lycopene	20	0.30 ± 0.21	0.43 ± 0.22	$P < 0.05$	0.15 ± 0.09	0.15 ± 0.08	N.S.
α-Carotene	20	0.04 ± 0.03	0.06 ± 0.05	N.S.	0.03 ± 0.02	0.03 ± 0.03	N.S.
β-Carotene	20	0.13 ± 0.08	0.17 ± 0.14	N.S.	0.08 ± 0.06	0.08 ± 0.07	N.S.
α-Tocopherol	20	7.00 ± 3.37	6.08 ± 2.75	N.S.	7.34 ± 5.19	6.91 ± 4.88	N.S.
γ-Tocopherol	20	1.68 ± 0.98	1.42 ± 0.69	N.S.	2.42 ± 1.71	2.22 ± 1.23	N.S.

[a]Values are expressed as μmol/g total lipid determined in the lipoprotein (mean ± SD); N.S., not significant; abbreviations as in Table 1; HDL–UC and LDL–UC, high and low density lipoprotein separation by ultracentrifugation.

TABLE 3
Carotenoids, α- and γ-Tocopherol Concentration in Whole Plasma: Fresh vs. Frozen Samples (μmol/g total plasma lipids)[a]

Vitamin	Fresh $n = 12$	Frozen[a] $n = 12$	*P* Value
Lutein/zeaxanthin	0.23 ± 0.14	0.19 ± 0.12	$P < 0.05$
β-Cryptoxanthin	0.11 ± 0.07	0.10 ± 0.05	N.S.
Lycopene	0.26 ± 0.13	0.31 ± 0.20	N.S.
α-Carotene	0.04 ± 0.03	0.06 ± 0.05	N.S.
β-Carotene	0.13 ± 0.09	0.15 ± 0.12	N.S.
α-Tocopherol	7.84 ± 2.48	8.78 ± 1.01	N.S.
γ-Tocopherol	2.04 ± 1.01	1.26 ± 0.53	$P < 0.01$

[a]Plasma samples were stored at −80°C for six months and thawed prior to analyses. Concentrations expressed as mean ± SD. Abbreviation as in Table 2.

Mean cholesterol concentration in the LDL–I was unchanged after storage of plasma samples (99.9 ± 26.7 vs. 102.1 ± 23.6 mg/dL, fresh vs. frozen). Mean cholesterol concentration in the HDL–P was significantly increased (51.1 ± 15.9 vs. 56.2 ± 14.4 mg/dL, fresh vs. frozen, respectively), and TG concentrations in HDL–P and LDL–I were increased (18.2 ± 6.4 and 20.7 ± 9.3 mg/dL vs. 25.2 ± 7.0 and 27.1 ± 14.5 mg/dL, fresh vs. frozen, respectively). Further, there was a significant increase in TG but not cholesterol in whole plasma.

Freeze/thawing of whole plasma had no effect on α-tocopherol and most carotenoids. Lutein/zeaxanthin and γ-tocopherol concentrations were greater in fresh whole plasma (Table 3).

A freeze/thaw effect similar to the effect observed for whole plasma was seen for LDL–I and HDL–P (Table 4). Lutein/zeaxanthin and γ-tocopherol were significantly lower in both fractions after freeze/thawing. Lycopene was decreased in LDL–I but not in HDL–P. There were no differences for other carotenoids and α-tocopherol.

DISCUSSION

Our goal in this study was to evaluate a method that permits fast and reliable plasma lipoprotein separation with subsequent accurate analyses of tocopherols and carotenoids in those fractions. To achieve this goal, immunoseparation and selective precipitation were used to isolate LDL and HDL, respectively. The traditional separation method, UC, was used as the reference.

To express lipid-soluble vitamins per gram lipid within each lipoprotein fraction, cholesterol and TG concentrations were determined after LDL and HDL separation. LDL cholesterol and LDL–TG concentrations were not different between the separation methods. This is consistent with the findings of McNamara *et al.* (5) who demonstrated that immunoseparation is appropriate for separation of LDL and subsequent cholesterol analyses. While immunoseparation has only recently been made commercially available, selective precipitation for the purpose of subsequent HDL–cholesterol determination has been used for many years in clinical laboratories as an alternative to UC (2). Selective precipitation in the present study was achieved with a medium molecular weight (M_r 50,000) dextran sulfate preparation, as introduced by Warnick *et al.* (6). Although this method previously has not been compared to UC, it was shown to compare well to precipitation with heparin–Mn^{2+}, a method demonstrated to have comparable accuracy to UC (6). The significantly higher HDL-P cholesterol and TG concentrations therefore remain unexplained. The higher lipid concentrations could be due to incomplete precipitation of apolipoprotein B particles or to higher recovery achieved with the precipitation method compared to UC. The fact that no turbidity, an indicator for insufficient apolipoprotein B precipitation, was observed in any supernate, argues against the former explanation.

The tocopherol concentrations in lipoproteins separated with the direct methods were comparable with UC separation. Therefore, we conclude that immunoseparation and selective precipitation is an appropriate method for the determination of α- and γ-tocopherols in LDL and HDL. There are few reports for tocopherol concentrations in lipoproteins separated by methods other than UC. Carcelain *et al.* (8) used selective precipitation to determine α-tocopherol in HDL. Those investigators redissolved the remaining precipitate for analyses of tocopherol in the VLDL–LDL fraction. They demonstrated that α-tocopherol concentrations in lipoproteins separated by precipitation were not significantly different compared to lipoprotein separation by UC. In a study by Clevidence and Lehman (4), HDL and VLDL–LDL fractions were separated using heparin affinity chromatography. Results were not directly compared to UC methods, but combined α-tocopherol and γ-tocopherol of HDL and VLDL–LDL fractions com-

TABLE 4
Carotenoid and α- and γ-Tocopherol Concentrations in LDL–I and HDL–P: Fresh vs. Frozen (μmol/g lipid)[a]

	LDL–I $n = 12$			HDL–P $n = 12$		
	Fresh	Frozen[a]	*P* value	Fresh	Frozen	*P* value
Lutein/zeaxanthin	0.26 ± 0.11	0.19 ± 0.06	$P < 0.05$	0.49 ± 0.11	0.37 ± 0.09	$P < 0.01$
β-Cryptoxanthin	0.07 ± 0.05	0.05 ± 0.03	N.S.	0.16 ± 0.12	0.12 ± 0.09	N.S.
Lycopene	0.29 ± 0.18	0.18 ± 0.11	$P < 0.05$	0.16 ± 0.09	0.15 ± 0.10	N.S.
α-Carotene	0.05 ± 0.02	0.04 ± 0.03	N.S.	0.04 ± 0.03	0.05 ± 0.03	N.S.
β-Carotene	0.15 ± 0.07	0.12 ± 0.09	N.S.	0.10 ± 0.07	0.09 ± 0.08	N.S.
α-Tocopherol	8.94 ± 2.22	8.27 ± 1.97	N.S.	11.23 ± 2.36	10.30 ± 2.27	N.S.
γ-Tocopherol	2.14 ± 0.92	1.20 ± 0.51	$P < 0.05$	2.95 ± 1.96	1.78 ± 0.71	$P < 0.05$

[a] Plasma samples were stored at −80°C for six months and thawed prior to lipoprotein separation. See Table 2 for abbreviations. Concentrations expressed as mean ± SD.

pared well to whole serum concentrations. In contrast to the methodologies of those studies (4,8), the combined use of precipitation and immunoseparation has the advantage that the precipitate does not have to be redissolved (8). Further, only LDL and not combined LDL–VLDL fractions, which is the case in precipitation or affinity column methods, is obtained with immunoseparation. The importance of this is that immunoseparation can be used with elevated TG levels and nonfasting samples, while high TG or postprandial concentrations of chylomicrons will interfere with the other separation methods (4,5,8).

Carotenoid concentrations in HDL separated by selective precipitation have not been reported previously. Results from the present study indicate that separation by precipitation presents a quick and efficient alternative for analyses of carotenoids in HDL. With the exception of lutein/zeaxanthin, carotenoid concentrations were not different between the methods. The reason for significantly higher lutein/zeaxanthin concentrations in HDL–P and LDL–I is not clear.

Similar to the situation for HDL, analyses of carotenoids in LDL have been performed in lipoproteins separated mainly by UC. Immunoseparation compared favorably to UC as an alternative method for subsequent carotenoid analyses in LDL. The two hydrocarbon carotenoids, α- and β-carotenes, and the hydroxycarotenoid, β-cryptoxanthin, were similar *via* the two methods.

Although desirable, analyses of fresh plasma samples are not always feasible. Instead, plasma samples may need to be frozen and stored for future analyses. It is therefore important to ensure that analytical procedures are adequate for samples previously frozen. We evaluated the effect of plasma storage at –80°C for 6 mon on the performance of the direct methods with subsequent lipid, carotenoid, and tocopherol analyses.

TG and cholesterol concentrations were significantly increased in HDL–P of frozen/thawed plasma samples. Results described in the literature regarding selective precipitation procedures applied to frozen/thawed samples are equivocal, suggest no change, a decrease or an increase in HDL–cholesterol concentrations after freezing (9–14). Bachorik *et al.* (14), using heparin–Mn^{2+} as precipitation reagent, showed an effect that was dependent on the initial HDL–cholesterol concentrations. Samples with low cholesterol concentrations (comparable to concentrations in the present study) tended to be higher, while samples with high HDL–cholesterol concentrations tended to be lower, after freeze/thaw. Increases after freeze/storage were reported for samples that were precipitated using phosphotungstic–$MgCl_2$ (13). In that study, interassay variability may have accounted for the effect (13). In the one study that has reported on the same precipitation reagent that we have employed, it was demonstrated that HDL–cholesterol was increased after storage in samples with plasma TG over 200 mg/dL, due to inadequate precipitation of apolipoprotein B particles (12).

In this study LDL–cholesterol concentrations in frozen samples were not different than cholesterol concentrations in fresh samples. This contrasts with the results of McNamara *et al.* (5) who reported a decrease in LDL cholesterol separated by immunoseparation after storage. Storage of plasma samples at temperatures of –70°C reportedly has no negative impact on carotenoid and tocopherol concentrations (15–18). Our results corroborate those findings for the majority of carotenoids and α-tocopherol in whole plasma. However, lower concentrations were observed for lutein/zeaxanthin and γ-tocopherol in whole plasma. These lower concentrations were apparently due to lower concentrations of these analytes in both HDL and LDL after storage (Table 4). Lycopene in LDL was decreased after storage. The remaining carotenoids and α-tocopherol in the LDL–I and HDL–P were not affected by freeze/thaw. While carotenoid stability in lipoproteins during storage has not previously been reported, the stability of α-tocopherol in LDL and HDL in the current study corroborates an earlier report (19).

In conclusion, the combined use of immunoseparation for LDL separation and selective precipitation for HDL isolation is an attractive accurate and reliable alternative to separation by UC for subsequent vitamin E and carotenoid analyses. Advantages of the combined method are that only a small sample volume is required, separation can be achieved within a short time, previously frozen plasma samples can be analyzed, and initial costs are minimal.

The present study included only normolipidemic subjects. Hypertriglyceridemia was previously associated with a positive bias for cholesterol measured in LDL separated by this immunoseparation technique (5). The impact of high plasma triglyceride concentrations on tocopherol and carotenoid analyses in LDL–I has not been evaluated in the present study.

Finally, it is important to note that although UC is often considered the "gold standard" for lipoprotein separation, UC lacks the necessary accuracy and precision to be considered a true reference method. UC is labor-intensive and time-consuming, and the method is also skill-dependent. There is a large margin for error resulting in imprecision and bias. Therefore, although we would like to see close agreement between the direct methods and UC, it is not absolutely necessary to validate a newer method against UC. In fact, with labile compounds, such as carotenoids, the faster, direct methods may, in fact, provide more accurate measurement of these analytes. Our data for LDL tocopherol and carotenoid concentrations represent a "worst case" scenario where measurements were made close to the detection limits of the assay. Still, the method provides good reproducibility and accuracy.

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Estimation of Polyunsaturated Fatty Acid Content in Lipids of Aquatic Organisms Using Thin-Layer Chromatography on a Plain Silica Gel Plate[1]

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ABSTRACT: We have designed a rapid method for the separation of polyunsaturated fatty acids (PUFA, ≥ trienes) from non-PUFA, and for estimation of total amounts of PUFA in lipids of aquatic organisms. Lipids from thirty-one species, including marine and fresh water fishes, shell fishes, marine algae, and other aquatic animals, and from terrestrial organisms, were transesterified with sodium methoxide in methanol. The resulting fatty acid methyl esters were separated by thin-layer chromatography on commercially available plain silica gel plates with a developing solvent of *n*-hexane/ethyl ether/acetic acid (95:5:1, by vol). All of the methyl esters from aquatic organisms tested separated into two spots, whereas those from terrestrial sources, except for linseed oil, showed a single unresolved spot. The upper and lower spots were scraped separately from the plate, and their fatty acid compositions were determined by gas–liquid chromatography. The lower spot was composed of PUFA having more than two double bonds, whereas components of the upper spot were saturated, monoenoic, and the greater part of the dienoic fatty acids. When the spots on the silica gel plate were stained with Coomassie brilliant blue, the amounts of PUFA in aquatic organisms could be estimated satisfactorily using a scanning densitometer.

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The physiological and nutritional importance of n-3 polyunsaturated fatty acids (PUFA, ≥ trienes) has been recognized in humans (1,2) and in other organisms (3–5). Icosapentaenoic acid, 20:5n-3 acid, reduces blood pressure and cholesterol levels in plasma (6,7), suppresses the production of eicosanoids of the 2 series, and increases the formation of eicosanoids of the 3 series (8–10). Docosahexaenoic acid, 22:6n-3 acid, is particularly high in content in mammalian tissues such as brain, retina, and sperm; the functional roles and biological essentiality of this acid have been elucidated (11–13). There is now a growing demand for marine oils, the main source of n-3 PUFA, for use in human foods, pharmaceuticals, and in industrial intermediates. Thus, there is a need for simple and adequate analytical methods for separation, identification, and evaluation of the resource oils.

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Abbreviations: GLC, gas–liquid chromatography; PUFA, polyunsaturated fatty acids; TLC, thin-layer chromatography.

Gas–liquid chromatography (GLC) and high-performance liquid chromatography are potent tools for separation of complex mixtures of natural fatty acids (14). Thin-layer chromatography (TLC) is widely used for identification or preparation of fatty acids and their derivatives. Mangold and Kammereck (15) and Wolfe *et al.* (16) demonstrated that acetoxymercurimethoxy derivatives of fatty acids can be resolved by TLC according to their degree of unsaturation. A more popular method is argentation TLC. Unsaturated fatty acids with both normal and substituted chains are clearly separated on a silica gel TLC plate impregnated with silver nitrate, which retards the migration of the more unsaturated species in relation to that of the more saturated ones (17,18). In these techniques, however, the procedure for the derivatization is tedious and the preparation of silver nitrate plates is expensive. Using ordinary and high-performance silica gel plates, Shantha and Ackman (19) demonstrated good separations of PUFA methyl esters from the saturates, monounsaturates, and diunsaturates.

We also made use of conventional TLC on plain silica gel and intended to design a simple and rapid method for differentiating lipids of aquatic organisms from those of terrestrial sources and for subsequent quantification of PUFA in the lipids by densitometry.

MATERIALS AND METHODS

Materials. Five marine fish: *Trachurus japonicus* (horse mackerel), *Chrysophrys major* (red sea bream), *Sardinops melanosticta* (sardine), *Limanda yokohamae* (mud lab), *Nibea mitsukurii*; seven freshwater fish: *Carassius carassius cuvieri* (crucian carp), *Tribolodon hakonensis* (dace), *Oncorhynchus mykiss*, *Anguilla japonica*, *Cyprinus carpio*, *Plecoglossus altivelis*, *Carassius carassius langsdorfii*; two shellfish: *Paphia undulata* (little clam), *Solen Gouldi* (razor clam), and meat tissues used for this experiment were purchased mainly from local retail stores. Seven algae: *Undaria pinnatifida* (wakame), *Ulva pertusa* (green laver), *Porphyra yezoensis*, *Ishige okamurai*, *Hizikia fusiformis*, *Colpomenia sinuosa*, and *Sargassum muticum*, were collected from the intertidal zone of northern Fukuoka. The lipids were extracted by homogenizing the tissue with chloroform/methanol (2:1, vol/vol) according to the procedure of Folch *et al.* (20). Plant

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seed oils were purchased from Wako Pure Chemical Industries (Osaka, Japan). Squid liver oil and other oils were donated by Dr. M. Furuichi, Laboratory of Fisheries Research Station, Kyushu University (Fukuoka, Japan).

Methylation. The lipid sample (~15 mg) in a screw-capped test tube was dissolved in benzene (0.5 mL) and 0.5 M-methanolic sodium methoxide (1 mL), and transmethylated at 80°C for 20 min (21).

TLC and densitometry. The fatty acid methyl esters from various organisms were spotted on the silica gel TLC plates which had been purchased from the E. Merck Co. (Darmstadt, Germany), Macherey-Nagel (Neumann-Neander-Strasse, Germany), Whatman Paper Ltd. (Maidstone, England), and Wako Pure Chemical Industries. The plates were activated at 110°C for 1 h before use, and developed with *n*-hexane/diethyl ether/acetic acid (95:5:1, by vol). The plates were sprayed with 50% (vol/vol) sulfuric acid and charred on a hot plate. Another set of plates dried by air-blowing were dipped into a staining solution consisting of 0.02% Coomassie brilliant blue (Fluka Chemicals AG, Buchs, Switzerland) in 20% methanol (22). After 30 min, the plates were removed from the staining solution, immersed in 20% methanol, and allowed to stand for 3 min with gentle agitation. The plates were removed from the stain removal solution, air dried, and spot intensities were measured using a scanning densitometer (CS-9300PC chromatoscanner; Shimadzu Co., Kyoto, Japan) at 600 nm of reflectance mode, subtracting blank readings from adjacent positions on the plate.

GLC. The TLC-spots or bands marked under ultraviolet light on the plate (Silica gel 60 F254; E. Merck Co.) were scraped off separately. The gel was extracted with chloroform/methanol (2:1, vol/vol), and the solvent was removed under reduced pressure. The resulting residue was analyzed by GLC, using a gas chromatograph (Shimadzu GC-14A; Shimadzu Co.) equipped with a flame-ionization detector and a capillary column (HR-SS-10, 30 m × 0.25 mm i.d.; Shinwakako Co., Kyoto, Japan). The column temperature was programmed for a linear increase of 4°C/min from 150 to 220°C. The methyl esters on the chromatograms were identified by conventional methods using the retention times of standards and the equivalent chain lengths, and by gas chromatography/mass spectrometry (QP-5000; Shimadzu Co.). Standard fatty acid methyl esters, 14:0, 16:0, and 18:0 were purchased from Wako Pure Chemical Industries, 20:0 and 22:0 were from GL Science Co. (Tokyo, Japan), 18:2n-6, 18:3n-3, and 20:4n-6 were from Sigma Chemical Co. (St. Louis, MO), and 20:5n-3 and 22:6n-3 were donated by Idemitsu Petro Chemical Co. (Tokyo, Japan). All of the reagents used in these experiments were of analytical grade.

RESULTS

Separation properties of fatty acid methyl esters on TLC. The fatty acid methyl esters prepared from various origins were separated on plain silica gel TLC plates. A typical chromatogram is shown in Figure 1. All of the esters from aquatic

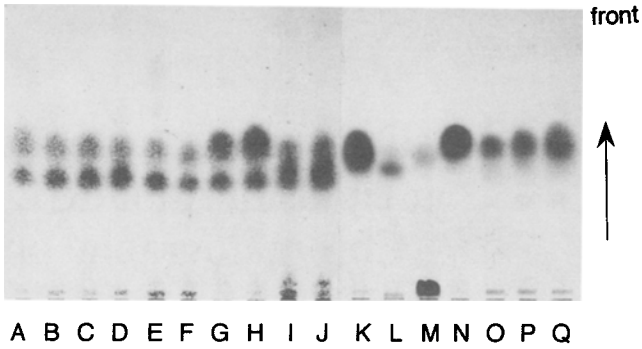


FIG. 1. Separation of fatty acid methyl esters prepared from lipids of aquatic and terrestrial organisms by thin-layer chromatography (TLC) on a plain silica gel plate. Conditions: TLC plate, Silica gel 60 F254 (E. Merck, Darmstadt, Germany); developing solvent, *n*-hexane/diethyl ether/acetic acid (95:5:1, by vol). The plate was sprayed with 50% (vol/vol) sulfuric acid and charred on a hot plate. A: horse mackerel, B: red sea bream, C: crucian carp, D: dace, E: little clam, F: razor clam, G: sea lion oil, H: sea snake oil, I: wakame, J: green laver, K: soybean oil, L: linseed oil, M: castor oil, N: olive oil, O: beef, P: pork, and Q: chicken.

organisms tested, including marine and freshwater fish, shell fish, marine algae, and some other aquatic animals such as sea snake and sea lion, were separated into two spots, whereas those from terrestrial organisms, including plant oils and meat tissues, showed one spot except for the ambiguous spots of linseed oil (Fig. 1L). These findings were confirmed using all of the commercially available silica gel plates used in this experiment.

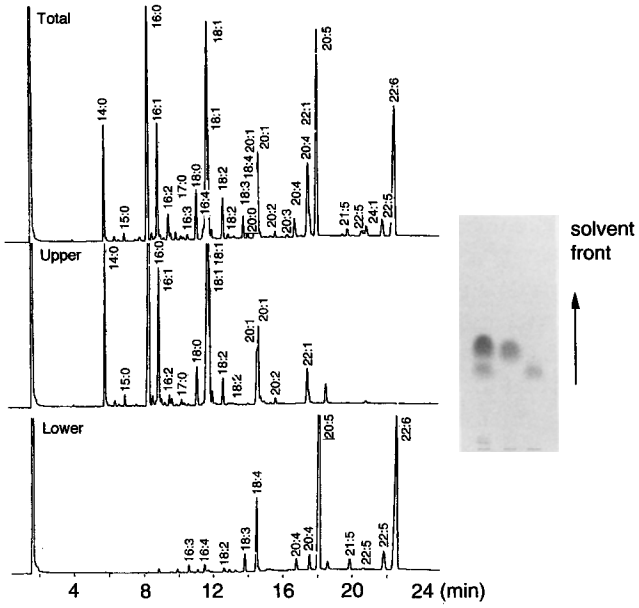


FIG. 2. Fatty acid analysis of the upper and lower spots on thin-layer chromatography (TLC) plates at methyl esters of squid liver oil by gas–liquid chromatography. Conditions: column, HR-SS-10 (0.25 mm × 30 m; Shinwa-kako, Kyoto, Japan); column temperature, 150 to 220°C (4°C/min); detector, flame-ionization detector. Conditions of TLC were as described in Figure 1. The plate was stained with 0.02% Coomassie brilliant blue. T: total methyl esters, U: upper spot, L: lower spot.

The upper and lower spots or bands marked under ultraviolet light on TLC plates were scraped off separately and analyzed on a capillary gas chromatographic column. As shown in the case of squid liver oil (Fig. 2), the upper spot consisted mainly of methyl esters of saturated and monoenoic acids, whereas the lower spot consisted of polyunsaturated acid esters which have more than two double bonds such as 16:4n-3, 18:3n-3, 18:4n-3, 20:4n-3, n-6, 20:5n-3, 21:5n-3, 22:5n-3, and 22:6n-3 acids. Fatty acids with two double bonds seemed to be distributed mainly in the upper spot with a part present in the lower spot.

The fatty acid compositions of organisms illustrated in Figure 1 are shown in Table 1. Aquatic animals have large amounts of long-chain PUFA such as 22:6n-3, 22:5n-3, 21:5n-3, 20:5n-3, and 20:4n-3, n-6 acids. Algae, wakame, or *Undaria pinatifida* contained relatively large amounts of 20:4n-6 and 20:5n-3 acids, but not 22:6 acid, and green laver *Ulva pertusa* contained large amounts of 18:3n-3, 18:4n-3, and small amounts of long-chain PUFA. On the contrary, only trace amounts of these PUFA were present in terrestrial animals and plant oils: the main components of the former were medium-chain, saturated, and monoenoic fatty acids such as 16:0, 16:1, 18:0, and 18:1 acids, and the latter comprises 16:0, 16:1, 18:1, 18:2, and 18:3 acids. Consequently, depending upon the composition and contents of PUFA, the methyl esters from aquatic organisms are separated clearly into two

TABLE 1
Fatty Acid Compositions of Lipids in Aquatic and Terrestrial Organisms Subjected to Thin-Layer Analysis in Figure 1 (area%)[a]

Sample	14:0	15:0	16:0	16:1 n-7	16:2 n-4	17:0	18:0	18:1 n-9	18:1 n-7	18:2 n-6	18:3 n-3	18:4 n-3
Horse mackerel	3.0	0.3	20.8	7.6	1.3	0.6	5.0	22.1	—	0.8	0.4	0.7
Red sea bream	1.9	0.5	21.8	4.8	0.9	0.6	6.1	15.1	—	0.9	0.2	0.3
Crucian carp	2.5	0.2	14.4	7.9	0.4	0.2	2.8	15.4	1.8	2.4	1.3	2.1
Dace	1.6	—	15.5	7.3	0.7	—	4.7	8.6	3.5	2.7	3.4	0.6
Little clam	1.3	0.3	11.3	4.2	0.6	0.7	5.3	2.5	4.1	0.5	0.7	4.7
Razor clam	2.5	0.5	10.6	7.0	0.9	0.5	3.5	1.1	3.7	0.8	0.7	3.3
Wakame	3.7	0.4	22.8	2.1	1.2	—	2.3	12.3	—	7.1	1.3	8.1
Green laver	0.8	0.1	22.5	2.7	—	1.0	9.3	10.7	—	8.0	15.4	7.6
Sea lion oil	3.3	0.2	12.7	5.7	0.6	0.3	4.6	27.5	—	3.0	0.6	6.1
Sea snake oil	1.8	0.4	24.0	3.9	0.5	0.6	6.5	34.4	—	0.9	0.4	1.7
Soybean oil	0.1	—	10.0	0.1	0.1	—	3.7	22.0	—	50.5	7.3	—
Linseed oil	—	—	4.2	—		—	2.6	16.4	—	13.3	62.6	—
Castor oil	—	—	1.0	—	—	—	1.2	3.9	—	4.8	0.7	0.8
Olive oil	—	—	9.7	0.8	—	—	2.7	72.1	—	4.3	0.8	0.5
Beef	1.8	0.5	19.3	4.4	—	—	13.0	35.8	—	4.2	1.1	—
Pork	1.2	—	21.0	3.9	—	—	7.7	40.2	—	11.3	0.6	—
Chicken	0.8	—	21.3	4.6	—	—	6.4	38.1	—	14.1	0.8	—

Sample	20:1 n-11	20:1 n-9	20:2 n-6	20:4 n-6	20:4 n-3	20:5 n-3	21:5 n-3	22:5 n-3	22:6 n-3	Other esters	≥ Triene (PUFA)
Horse mackerel	2.2	—	0.3	1.4	0.7	8.3	0.5	2.3	10.4	11.3	25.1
Red sea bream	2.3	0.3	0.5	2.7	1.1	4.7	0.3	3.4	22.6	9.0	35.4
Crucian carp	2.5	—	1.7	2.2	2.5	9.5	0.7	2.9	14.7	11.9	36.3
Dace	1.2	0.2	0.8	5.1	1.7	12.8	0.5	3.8	12.0	13.3	40.5
Little clam	1.3	2.4	1.8	3.0	1.9	14.6	1.6	3.3	13.3	20.6	43.2
Razor clam	0.9	1.9	1.9	3.5	4.4	17.9	2.1	2.3	7.4	22.6	41.6
Wakame	—	—	0.3	13.0	1.3	8.8	0.3	—	0.4	14.6	33.6
Green laver	1.4	—	0.9	1.6	1.5	2.3	—	3.2	0.8	10.2	34.2
Sea lion oil	3.1	—	0.8	1.1	3.1	5.0	0.9	3.2	6.2	12.0	26.3
Sea snake oil	0.3	0.1	0.5	1.7	0.5	1.1	—	2.0	6.0	12.7	13.4
Soybean oil	—	—	—	—	—	—	—	—	—	6.2	7.3
Linseed oil	—	—	—	—	—	—	—	—	—	0.9	62.6
Castor oil	—	—	0.5	—	—	—	—	—	—	87.1	1.5
Olive oil	0.6	0.1	—	—	—	—	—	—	—	8.4	1.3
Beef	—	—	—	1.8	—	—	—	—	—	18.1	5.2
Pork	1.2	—	1.6	1.6	—	—	—	1.2	0.6	7.9	4.0
Chicken	0.6	—	—	3.3	0.7	—	—	1.8	1.9	5.6	8.5

[a]PUFA, polyunsaturated fatty acids.

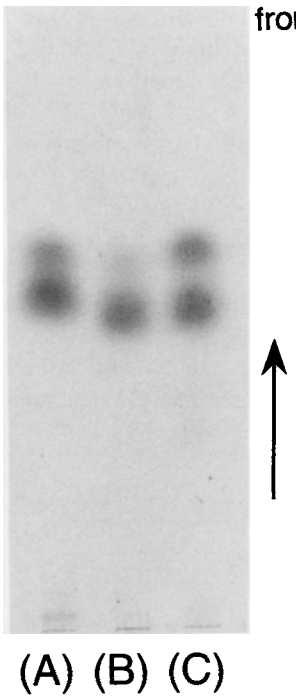


FIG. 3. Separation behaviors of fatty acid methyl ester from linseed oil on a silica gel thin-layer plate. Conditions were as described in Figure 1. (A): methyl ester of linseed oil, (B): same weight mixture of (A) and methyl docosahexaenoate, (C): same weight mixture of (A), methyl docosahexaenoate, and methyl oleate.

spots on the TLC plate, whereas fatty acid methyl esters from terrestrial organisms which have little PUFA showed only the one upper spot. The esters from the algae are less separable because they had little 22-carbon PUFA. The separation behaviors of the fatty acid esters of linseed oil, which has a high content of 18:3n-3 acid (62.6%) and little of long-chain PUFA, were studied in further detail. As shown in Figure 3, the total fatty acid esters were separated into very close double spots (Fig. 3A). By addition of an equal amount of 22:6n-3 acid ester, the lower spot of 18:3n-3 acid ester showed less mobility and formed one spot with 22:6n-3 acid ester (Fig. 3B). A same weight mixture of the linseed oil, 22:6n-3, and 18:1n-9 esters showed clear subfractionation (Fig. 3C), similar to that found on aquatic organisms in Figure 1.

Densitometric analysis of PUFA. As described above, the methyl esters of PUFA of aquatic organisms, especially of fish and fish oils, are clearly separated from more saturated ones. Using this characteristic, the content of PUFA in aquatic organisms was estimated following staining with Coomassie brilliant blue. The results of grouping analyses of fatty acids by densitometry are summarized in Table 2. The amounts obtained were in good agreement with those obtained by GLC, though lower amounts of PUFA in fish lipids and higher amounts of PUFA in algae than those determined by GLC were estimated by densitometry. The amount of PUFA can be roughly estimated by eye after conventional charring with sulfuric acid on a hot plate. However, this charring method could not be applied for densitometric determination, because the charring intensities of the lower spot (PUFA) and the upper spot were quite different; the upper spot resisted charring with sulfuric acid.

DISCUSSION

As shown in Table 1, the fatty acid compositions of lipids of aquatic organisms are complex. Factors including season, water temperature, nature of fats in their diets, maturity, and gender affect the makeup of the distribution patterns. As a result, the composition and contents of the fatty acids may vary not only from species to species, but to an even greater extent from one fish to another even of the same species. However, because aquatic organisms constitutionally contain relatively large amounts of long-chain PUFA, their fatty acid methyl esters are separated into two spots on the plain silica gel plate, as seen in Figure 1. Thus, using the characteristic behavior on the TLC plate, one can distinguish fish oils from terrestrial oils containing little PUFA, and can apply the method to the preparation and estimation of PUFA containing more than two double bonds. Shantha and Ackman (19) recommended use of their TLC method for enrichment of PUFA in foods and marine lipids. They detected amounts down to 1% or less of PUFA spot or band on the TLC plate, although the distribution behaviors of the trienes such as linolenate were different from those of our method. They reported that the dienes distributed to the upper and the trienes to both the upper and lower spots.

TABLE 2
Grouping Analyses of Fatty Acids in Aquatic Organisms by Densitometry and GLC[a]

	TLC		GLC		
Lipids	Upper spot non-PUFA (%)	Lower spot PUFA	Dienes+ non-PUFA (%)	≥ Triene PUFA	Lipid content (%)
Sardine	57.1 ± 0.6	42.9 ± 0.6	55.3 ± 0.4	44.7 ± 0.4	2.2
Horse mackerel	82.1 ± 0.8	17.9 ± 0.8	74.7 ± 0.9	25.4 ± 0.6	6.1
Squid liver oil	74.7 ± 2.4	25.3 ± 2.4	68.1 ± 0.9	31.9 ± 0.9	—
Crucian carp	69.8 ± 2.9	30.2 ± 2.8	62.9 ± 0.6	37.1 ± 0.6	3.5
Dace	67.2 ± 1.1	32.8 ± 1.1	63.3 ± 1.1	36.7 ± 1.1	1.0
Wakame	59.0 ± 1.1	41.1 ± 1.1	61.4 ± 1.0	38.7 ± 1.0	2.2
Green laver	56.0 ± 3.5	44.0 ± 3.5	62.7 ± 1.6	37.3 ± 1.6	1.3

[a]The results are expressed as the mean value with standard deviation for triplicate determinations, TLC, thin-layer chromatography; GLC, gas–liquid chromatography. See Table 1 for other abbreviation.

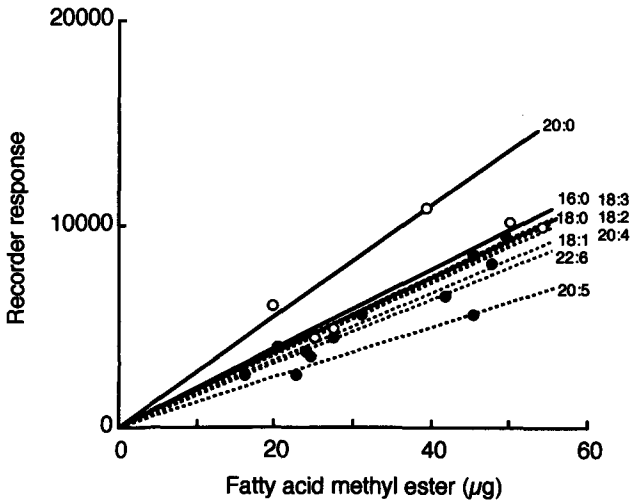


FIG. 4. Staining intensities of authentic fatty acid methyl esters. After thin-layer chromatographic separation under the same conditions as described in Figure 1, the spots were stained with 0.02% Coomassie brilliant blue in 20% methanol for 30 min. See text for details; —○—, saturated ester; – –●– –, unsaturated ester.

Staining with Coomassie brilliant blue is a practical technique. Some discrepancy of analytical values between GLC and densitometry in Table 2 may be reflected in the fundamental difference of the analytical principles of the two methods. Another reason may be attributed to the intrinsic stainability of esters by Coomassie brilliant blue (Fig. 4). Generally, the longer the fatty acid chain and the fewer the double bonds, the more intense is the stainability. However, because the main fatty acids in lipids of aquatic organisms have a similar stainability, the method can be used for estimation of PUFA contents in aquatic organisms.

Oshima *et al.* (23) studied chromatographic behavior or free fatty acid on Iatroscan TLC-FID Chromarods. They observed similar separation of free PUFA from non-PUFA. If the method is applicable to separation of fatty acid methyl esters, better agreement of the measured value with those of GLC may be expected.

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Increased Hepatic Synthesis and Accumulation of Plasma Apolipoprotein B100 in Copper-Deficient Rats Does Not Result from Modification in Apolipoprotein B mRNA Editing

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ABSTRACT: Experimentally induced copper deficiency in the rat results in increased plasma apolipoprotein B100 (apo B100) concentration in association with increased hepatic apo B100 synthesis. This enhancement of apo B100 synthesis and plasma accumulation accounts for the rise of plasma low density lipoprotein in these animals. In the present study, we have investigated if the selective increase in hepatic apo B100 synthesis is accounted for by changes in apo B mRNA editing. Reverse transcription coupled with polymerase chain reaction amplification and primer extension analysis of apo B cDNA revealed no differences in apo B mRNA editing in either the liver or small intestine between control and copper-deficient rats. We speculate that the increase in apo B100 synthesis in the liver of copper-deficient rats reflects posttranslational alterations in gene expression accompanying changes in very low density lipoprotein assembly and secretion.
Lipids 31, 433–436 (1996).

Dietary copper is often low in the Western diet, and dietary copper deficiency has been speculated to be involved in the etiology of atherosclerosis (1). Studies in copper-deficient rats have pointed to several potential mechanisms whereby copper deficiency may contribute to atherosclerosis susceptibility (1). It has been shown, for example, that copper deficiency induces hypercholesterolemia (2), affects oxidative defense (1), increases the susceptibility of lipoproteins to peroxidation (3), and impairs prostaglandin metabolism (4). The hypercholesterolemic effects of copper deficiency in various experimental animal models is well documented (2), and has been suggested to arise from an increased rate of hepatic cholesterol synthesis (5). It has been proposed that this increased cholesterol synthesis may be due to the elevated hepatic glutathione and in turn elevated 3-hydroxy-3-methyl-glutaryl CoA reductase activity (6). Copper deficiency-induced hypercholesterolemia is also associated with changes in lipoprotein distribution and composition (7–9). This hypercholesterolemia mainly results from an increase in cholesterol concentration in high density lipoprotein and low density lipoprotein fractions in conjunction with increased plasma apolipoprotein (apo) E and apo B concentrations (9,10). In addition, density gradient ultracentrifugation and immunoblot analysis of isolated lipoproteins showed an increase in apo B100 abundance in copper-deficient rats as compared to controls (10).

In regard to the sources of apo B containing lipoproteins in mammals, it is well established that there is organ-specific production of two distinct forms (apo B100 and apo B48), which are products of a common gene (11). In humans, apo B100 is synthesized in the liver, and apo B48 in the small intestine. In rats, both forms of apo B are synthesized and secreted by the liver. Apo B48 mRNA is produced from apo B100 mRNA by a unique mechanism known as RNA editing. Apo B mRNA editing involves a site-specific C to U cytidine deamination reaction which is mediated by an enzyme complex containing a catalytic subunit, apobec-1 (11–13). In view of the finding that copper deficiency selectively increases hepatic apo B100 synthesis (10), the present study was designed to determine whether this effect is mediated through alterations in apo B mRNA editing.

MATERIALS AND METHODS

Animals and diets. Weanling male Wistar rats (IFFA-Credo, L'Arbresle, France) were randomly divided into copper-deficient and control groups. Rats were housed in wire-bottomed cages in a temperature-controlled room (22°C) with 12 h light–dark cycle and were pair-meal-fed the indicated diet for 6 wk. The appropriate weight of food (as determined below) was delivered to each group of animals at regularly spaced, timed intervals, three times each day. Food consumption was determined directly by weighing the residual from each feeding cycle and continuously adjusting the amount delivered to ensure that both groups consumed identical portion sizes. The semipurified diet contained (g/kg) casein 200, sucrose 650, corn oil 50, alphacel 50, DL-methionine 3, choline bitartrate

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Abbreviations: Apo, apolipoprotein; RT–PCR, reverse transcriptase–polymerase chain reactions.

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2, modified AIN-76 mineral mix 35, and AIN-76 A vitamin mix 10 (ICN; Biomedicals, Orsay, France). Cupric carbonate was omitted from the AIN-76 mineral mix in the copper-deficient diet. The copper concentrations of the diets were 0.4 mg/kg (deficient) and 6 mg/kg (control). Animals were anesthetized with sodium pentobarbital (40 mg/kg body weight) 15 h following the last meal and were then exsanguinated. The final body weight of rats was 287 ± 3 and 246 ± 5 g (n = 6 per group; $P < 0.001$) for the control and copper-deficient groups, respectively. Reduced plasma copper concentrations, assessed by atomic absorption spectrometry (Perkin Elmer 560; Perkin Elmer, Norwalk, CT) were found in copper-deficient rats as compared to control animals (0.6 ± 0.1 vs. 14.2 ± 0.5 μmol/L).

Analysis of apo B isomorph distribution. Lipoprotein fractions (d < 1.063 g/L) were isolated from plasma by ultracentrifugation 18 h at 100,000 × g. Samples of isolated lipoproteins were submitted to 4–20% SDS–polyacrylamide gel electrophoresis (Biorad, Ivry Sur Seine, France). The samples were subsequently electroblotted to nitrocellulose membranes (Hybond C Super; Amersham, Bucks., England). Immunodetection was performed using a rabbit antirat low density lipoprotein antibody and enhanced chemiluminescence (Amersham). Parallel analyses were performed on these aliquots using a rabbit antirat apo E antiserum. Quantification of signals was performed by laser densitometry.

Analysis of apo B mRNA editing. Total cellular RNA from liver and small intestine was isolated using the guanidinium/phenol/chloroform method (14). RNA integrity was assessed by agarose gel electrophoresis and visualization of 18S and 28S ribosomal RNA by ethidium bromide staining. Aliquots of total RNA (2 to 10 μg) were DN-ased as described (15) and approximately 100 ng used for reverse transcriptase–polymerase chain reaction (RT–PCR). Reverse transcription was performed at 70°C for 15 min using a downstream rat apo B antisense primer (ND3) and 5u rTth (# N808-0097; Perkin-Elmer, Cetus, CT) (15,16). Following reverse transcription, 80 μL (final volume) of PCR buffer was added along with a rat apo B sense primer (ND1) (15). PCR was performed for 30 cycles of 30 s at 95°C, 1 min at 55°C, 1.5 min at 72°C, with a final 10-min extension. For each RNA sample, a negative control was run to check for DNA contamination using rTth and leaving the sample on ice during reverse transcription. Additionally, each reaction contained a tube with all above buffers and enzymes but without RNA to exclude PCR product contamination. Reaction material (15–20 μL) was analyzed by agarose electrophoresis to demonstrate the expected 275 base pair apo B cDNA amplification product. RT–PCR products were purified using spin columns and apo B cDNA editing assayed by primer extension using an end-labeled oligonucleotide antisense primer (BT7) and T7 DNA Polymerase, as detailed previously (15). The dried gel was exposed to Kodak XAR film (Rochester, NY) at –80°C with enhancing screens. Autoradiographs at comparable exposures were scanned using a laser densitometer. We have previously validated the linearity of this assay between 1 and 99% editing of a synthetic RNA template (15).

Statistical analysis. Results were expressed as mean ± SEM. The statistical significance of differences between means was assessed by Student's t- and Mann-Whitney tests.

RESULTS AND DISCUSSION

Previous studies from our group have shown that experimentally-induced copper deficiency in the rat results in an increased apo B100 concentration in the plasma in association with increased hepatic apo B100 synthesis (10). The present work confirms these findings as evidenced by the increased ratio of apo B100/apo B48 in plasma of copper-deficient rats as compared to their pair-meal fed controls (Fig. 1A). Additionally, as inferred by Western blot analysis, apo E abundance was increased in the d < 1.063 g/L fraction (Fig. 1B), findings which confirm our previous demonstration of increased plasma apo E concentrations in copper-deficient rats (9). Since a selective increase in hepatic apo B100 synthesis was demonstrated in copper-deficient rats, we determined whether changes in apo B mRNA editing in the liver could explain this modification. The data reveal no significant differences in the extent of apo B mRNA editing in either the liver or small intestine (Table 1 and Fig. 2).

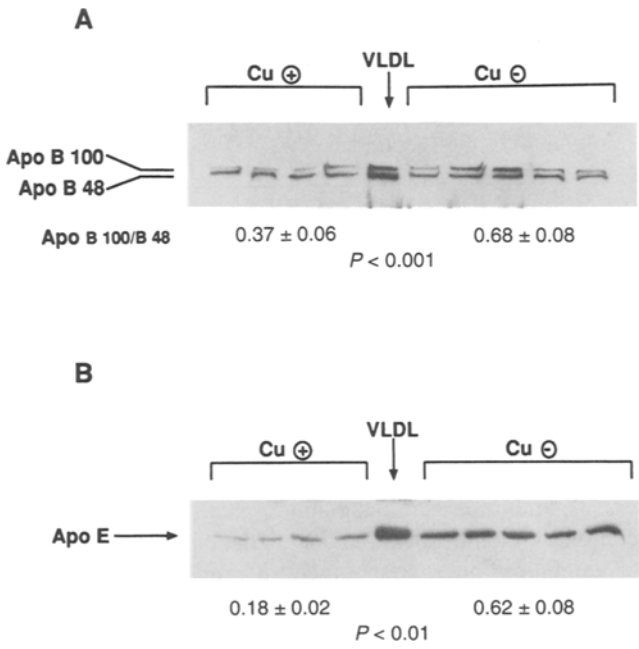


FIG. 1. Immunoblot analysis of plasma (A) apolipoprotein (apo) B isoforms and (B) apo E. Aliquots of lipoprotein fractions (d < 1.063 g/L) isolated from control and copper-deficient rats were electrophoresed through a 4–20% SDS–polyacrylamide gel electrophoresis slab gel and transferred to nitrocellulose membrane. Immunodetection was performed using either a rabbit anti-rat (A) low density lipoprotein or (B) anti-apo E antibody and enhanced chemiluminescence; VLDL, very low density lipoprotein.

TABLE 1
Apolipoprotein B mRNA Editing (%TAA) in the Liver and Small Intestine of Control and Copper-Deficient Rats[a]

	Experimental group	
	Control	Copper-deficient
Liver	73 ± 6	66 ± 8
Jejunum	92 ± 1	95 ± 1
Ileum	95 ± 1	96 ± 1

[a]Results are given as means (±SEM) of three to four individual measurements per group. There was no significant differences between the two groups of rats ($P > 0.05$); TAA = the proportion of edited apolipoprotein B cDNA.

Regulation of apo B synthesis and secretion can theoretically occur at a number of levels, including changes in apo B mRNA abundance, apo B mRNA editing, and modifications in intracellular degradation of newly synthesized apo B (16–20). Our previous studies (10,21), taken together with the present work, show that copper deficiency affects apo B synthesis without modification in either apo B mRNA abundance or posttranscriptional RNA editing. Thus, we speculate that the increase in apo B100 synthesis in the liver of copper-deficient rats reflects posttranslational alterations in apo B gene expression, presumably including intracellular degradation and secretion, associated with the changes in very low density lipoprotein assembly and secretion. Previous work has shown that copper deficiency enhances the hepatic synthesis of cholesterol (5), fatty acids, and phospholipids (22), suggesting that these alterations may lead to an increased secretion of triglyceride-enriched hepatic very low density lipoprotein. Nevertheless, the precise mechanism by which copper deficiency selectively enhances apo B100 synthesis remains to be elucidated.

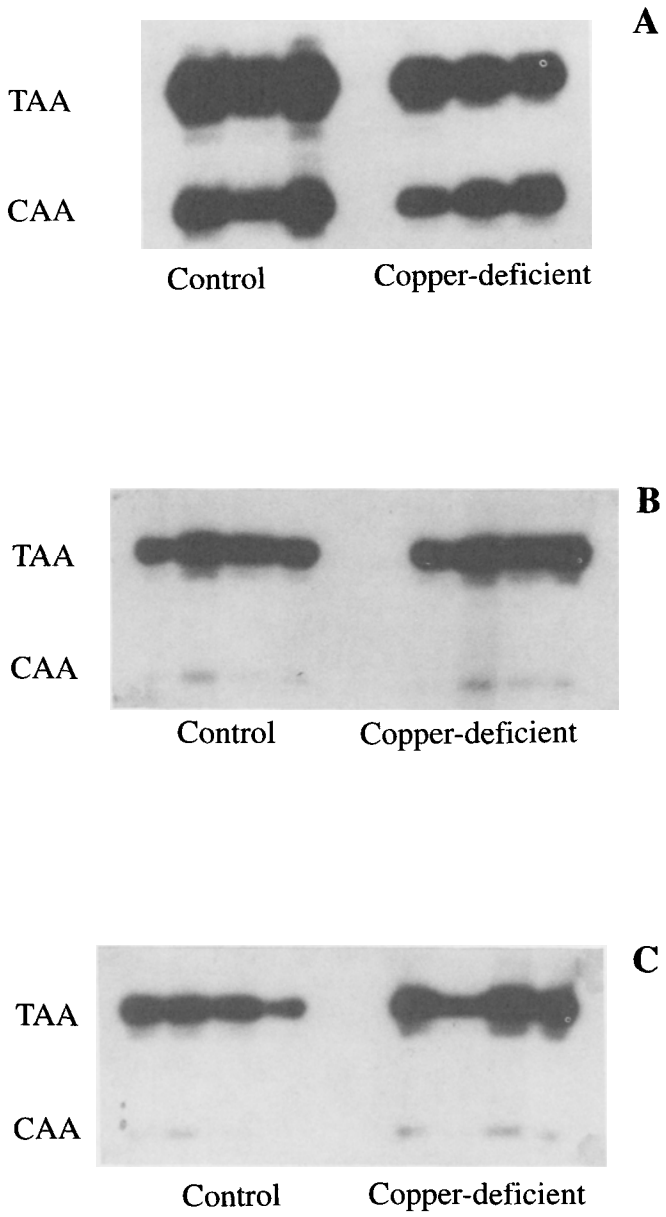


FIG. 2. Apolipoprotein (apo) B mRNA editing in control and copper-deficient rats, determined by reverse transcriptase–polymerase chain reaction (PCR) and primer extension analysis. Purified PCR products were subjected to primer extension analysis to demonstrate the proportion of edited (TAA) vs. unedited (CAA) apo B cDNA: (A) liver, (B) jejunum, (C) ileum.

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Postnatal Changes in Activation of Polyunsaturated Fatty Acid, Acylation of Lysophosphoglyceride, and Phosphorylation of Diacylglycerol in Cortical Gray Matter of Rat Cerebrum

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ABSTRACT: Activation of polyunsaturated fatty acid, acylation of lysophosphoglyceride, and phosphorylation of diacylglycerol in postnatal developing rat cerebrum were studied *in vitro*, using cortical gray matter homogenates. The rate of n-3 and n-6 fatty acid activation was maximal at ten days after birth and minimal at adulthood. Acylation of lysophosphoglyceride was most active at the neonatal stage and gradually decreased with age. In addition, the amount of phosphatidic acid formed from 1, 2-dioleoyl-glycerol was also maximum at the neonatal stage, and then gradually decreased. These results suggest that the specific activity of glycerolipid synthesis in cortical gray matter declines during postnatal development.

Lipids 31, 437–440 (1996).

During development, the brain actively accumulates long-chain polyunsaturated fatty acids (PUFA) in many species (1), and the amount of phospholipid increases with age (2). Long-chain PUFA is preferentially located in the *sn*-2 position of glycerophospholipid and plasmalogen (3,4). This selective incorporation of long-chain PUFA to the *sn*-2 position of glycerophospholipid is known to be regulated by a deacylation–reacylation mechanism (5,6). Acyl-CoA synthetase and acyl-CoA:lysophosphoglyceride (LPG) acyltransferase catalyze CoA + ATP-dependent acylation, which is one of three types of reactions related to deacylation–reacylation in the brain (7). Previous studies on the acylation of LPG in the developing brain (8,9) have not addressed PUFA. In this study, acyl transfer by acyl-CoA:LPG acyltransferase in the cortical gray matter of developing rat cerebrum was investigated, in addition to PUFA-activation by acyl-CoA synthetase.

Changes in diacylglycerol (DG) kinase activity in cortical gray matter were also investigated. The brain contains several times more phosphatidic acid (PA) than the liver or retina, and the PA content in mouse adult brain is much greater than that in neonatal brain (10). The formation of PA by DG kinase in rat cerebral cortex occurs at a much greater rate than the acylation of *sn*-glycerol-3-phosphate (11). It has been suggested that DG kinase makes a considerable contribution to the biosynthesis of PA in the brain. Both PA and DG are intermediates in glycerophospholipid synthesis (12). PA is converted to phosphatidylinositol through cytidine diphosphate DG, and DG is a substrate for the synthesis of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and triacylglycerol. It has been suggested that DG kinase might regulate the levels of DG and/or PA. Currently, there is no evidence that DG kinase activity changes during development.

MATERIALS AND METHODS

Animals. Female and male Wistar rats (Funabashi Farm, Funabashi, Chiba, Japan) were fed a semipurified diet containing 10% soybean oil (13), and mated at 12 wk of age. Pups were weaned at 3 wk of age and maintained *ad libitum* on the same diet until they were 26-weeks-old. Cerebral cortices were obtained from newborn, 10-day-old, 20-day-old, 30-day-old, 16-week-old, and 26-week-old rats soon after decapitation, and kept at −80°C until use.

Materials. [1-^{14}C]Linoleic acid (2.0 GBq/mmol), [1-^{14}C]-linolenic acid (2.0 GBq/mmol), [1-^{14}C]arachidonic acid (2.0 GBq/mmol), [1-^{14}C]eicosapentaenoic acid (1.9 GBq/mmol), [1-^{14}C]docosahexaenoic acid (1.9 GBq/mmol), and [1-^{14}C]-arachidonyl-CoA (2.1 GBq/mmol) were purchased from New England Nuclear (Boston, MA). [γ-^{32}P]ATP (110 GBq/mmol) was purchased from Amersham (Amersham, United Kingdom). Radio-labeled fatty acids and ATP were diluted with unlabeled fatty acids (Funakoshi, Tokyo, Japan) and ATP (Oriental Yeast, Tokyo, Japan). Bovine serum albumin (BSA; essentially fatty-acid free, Fraction V) was obtained from Sigma (St. Louis, MO). 1,2-Dioleoyl-*sn*-glycerol (18:1/18:1 DG), 1-stearoyl-2-arachidonoyl-*sn*-glycerol (18:0/20:4 DG),

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Abbreviations: BSA, bovine serum albumin; DG, diacylglycerol; 18:1/18:1 DG, 1, 2-dioleoyl-*sn*-glycerol; 18:0/20:4 DG, 1-stearoyl-2-arachidonoyl-*sn*-glycerol; LPC, lysophosphatidylcholine; LPEP, lysophosphatidylethanolamine plasmalogen; LPG, lysophosphoglyceride; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid.

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lysophosphatidylcholine (LPC) (porcine liver), and lysophosphatidylethanolamine plasmalogen (LPEP) (bovine brain) were purchased from Funakoshi. Other chemicals were purchased from Wako Pure Chemical (Osaka, Japan).

Preparation of homogenates. Gray matter, obtained from the cerebral cortex, was homogenized in 0.15 M KCl/20 mM Tris-HCl buffer solution (pH 7.4) and centrifuged at 700 g for 5 min. The supernatant was used for the enzyme assays. The protein content of the supernatant was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

Enzyme assays. Fatty acid activation was assayed according to the procedure of Reddy and Bazan (14). The acylation of LPG was measured by a modification of the procedure described by Fisher and Rowe (15). A final volume of 150 μL of 100 mM Tris-HCl buffer solution (pH 7.4) contained 2 nmol 20:4n-6-CoA (4.2 kBq) [or 2 nmol 22:6n-3 (1.0kBq), 200 nmol ATP, 50 nmol $CoANa_3$, 4 μmol $MgCl_2$, 7.5 μmol $NaHCO_3$, and 4 nmol BSA], 15 nmol LPC (or LPEP), and 100 μg homogenate protein. The reaction was started by adding homogenate solution and stopped by adding 3.65 mLof chloroform/methanol/0.1 N HCl (100:200:65, by vol) after incubation at 37°C for 1–3 min. A blank assay was carried out without LPG. Lipid extract was obtained from the reaction mixture using the method of Bligh and Dyer (16), and applied to thin-layer chromatography (17). The PC or PE on Silica Gel 60 aluminum plate (Merck, Darmstadt, Germany) viewed under iodine vapor was cut out, transferred to a scintillation vial containing scintillation fluid, and counted in an Aloka LC903 (Aloka, Tokyo, Japan). The phosphorylation of DG was assayed according to the procedure of Kanoh and Ohno (18).

Statistical analysis. Differences between groups were analyzed by the Mann-Whitney U-test when the P value in the Kruskal-Wallis test was less than 0.05.

RESULTS AND DISCUSSION

Reddy and Bazan (14) reported that the activation of saturated fatty acid (16:0) and PUFA (18:2n-6, 20:4n-6, and 22:6n-3) occurred at a maximum rate in mouse whole brain at 15 d of age. We also observed that PUFA-activation in rat brain was enhanced in the middle of the suckling period. Table 1 shows the amounts of acyl-CoA formed from n-6 fatty acids by whole brain homogenates. The specific activities of both 18:2n-6 and 20:4n-6 CoA synthetase in newborns were significantly lower than those in 10-day-old rats, while no significant differences were observed between newborns and 30-day-old and 26-week-old rats. Furthermore, a rapid increase in the rate of PUFA-activation up until 10 d after birth was observed in cortical gray matter (Table 2), which contains a high concentration of PUFA (19). The amount of acyl-CoA formed was maximum at 10 d, and minimum at 16 and 26 wk after birth, regardless of the substrate fatty acid. PUFA-activation in adult rats occurred at a considerably slower rate than that in younger rats, although the decline in PUFA-activation observed in whole brain was not remarkable. The specific activity of acyl-CoA synthetase in cortical gray matter changed drastically with age. The results of this study suggest that the use of PUFA in cortical gray matter is less active in adult rats than younger rats.

TABLE 1
Activation of n-6 Fatty Acids by Whole Brain Homogenates[a]

Fatty acid	0 d	10 d	30 d	26 wk
	(nmol/min/mg of protein)			
18:2n-6	3.82 ± 0.22[a]	4.93 ± 0.67[b]	4.73 ± 0.93[a,b]	4.06 ± 0.29[a,b]
20:4n-6	3.48 ± 0.44[a]	4.64 ± 0.49[b]	3.76 ± 0.57[a,b]	2.90 ± 0.41[a]

[a]Values (means ± SD of five litters or five rats) with different superscript letters (a,b) are significantly different ($P < 0.05$).

It has been demonstrated that acyl-CoA:LPG acyltransferase in rat brain microsomes shows an acceptor preference in the descending order of 1-acyl-glycero-3-phosphocholine, 1-acyl-glycero-3-phosphoethanolamine, and 1-alk-1′-enyl-glycero-3-phosphoethanolamine (plasmalogen type), and seems to be acylated by 18:1n-9 and 20:4n-6 rather than by 22:6n-3 (7). We also observed that the rate of transference of 20:4n-6 to LPEP (1-alkenyl) was considerably lower than that of LPC (1-acyl) by cortical gray matter homogenates. It has been shown that the remodeling of ether-linked phospholipid is catalyzed by direct transfer of a fatty acid between two molecules of glycerophospholipid rather than by acyl-CoA:LPG acyltransferase (5,7). In inflammatory cells, 20:4n-6 is initially incorporated into PC and subsequently transferred to PE plasmalogen (5). In the present study, the rate of the transference of 20:4n-6 to both LPC and LPEP was maximum in newborns (Table 3), and the radioactivity of the PC fraction was also maximum in newborns in an assay with-

TABLE 2
Activation of n-3 and n-6 Fatty Acids by Cortical Gray Matter Homogenates[a]

Fatty acid	0 d	10 d	30 d	16 wk	26 wk
			(nmol/min/mg of protein)		
18:2n-6	3.44 ± 0.31[a]	5.08 ± 1.08[b]	4.21 ± 0.65[a]	1.72 ± 0.24[c]	1.61 ± 0.23[c]
18:3n-3	3.16 ± 0.16[a]	4.96 ± 0.98[b]	3.53 ± 0.54[a]	1.48 ± 0.23[c]	1.40 ± 0.17[c]
20:4n-6	3.11 ± 0.42[a]	5.60 ± 0.78[b]	3.16 ± 0.55[a]	1.41 ± 0.27[c]	1.06 ± 0.20[c]
20:5n-3	3.73 ± 0.28[a]	6.14 ± 1.09[b]	4.10 ± 0.58[a]	1.85 ± 0.34[c]	1.44 ± 0.25[c]
22:6n-3	2.11 ± 0.15[a]	3.85 ± 0.74[b]	2.46 ± 0.35[a]	1.21 ± 0.27[c]	1.11 ± 0.16[c]

[a]Values (means ± SD of five litters or five rats) with different superscript letters (a–c) are significantly different ($P < 0.05$).

TABLE 3
Acyltransfer to Lysophosphoglyceride by Cortical Gray Matter Homogenates[a]

Donor	Acceptor	0 d	10 d	30 d	16 wk	26 wk
				(nmol/min/mg of protein)		
20:4-CoA	LPC	2.05 ± 0.17[a]	1.96 ± 0.21[a]	1.64 ± 0.17[b]	1.32 ± 0.15[c]	1.40 ± 0.16[c]
20:4-CoA	LPEP	0.19 ± 0.03[a]	0.16 ± 0.02[a,b]	0.15 ± 0.02[b]	0.16 ± 0.01[b]	0.16 ± 0.01[b]
22:6	LPEP	0.10 ± 0.008[a]	0.10 ± 0.01[a,b]	0.09 ± 0.01[a,b]	0.08 ± 0.006[a,b]	0.07 ± 0.02[b]

[a]Values (means ± SD of five litters or five rats) with different superscript letters (a–c) are significantly different ($P < 0.05$); LPC, lysophosphatidylcholine; LPEP, lysophosphatidylethanolamine plasmalogen.

out exogenous LPG (data not shown). Furthermore, the activity of acylation showed an age-dependent decrease, regardless of whether 20:4n-6-CoA or 22:6n-3 was used as a substrate. The incorporation of 22:6, which is catalyzed by acyl-CoA synthetase and acyl-CoA:LPG acyltransferase, also occurred at a maximum rate in newborns and was hardly affected by the change in the activity of acyl-CoA synthetase with age. It has been shown that PC is formed from 18:1 and LPC at a maximal rate by cortical gray matter homogenates of rhesus monkey at the neonatal stage (8). In rat whole brain, the activity of acylating lyso-PE (1-acyl, 1-alkyl, and 1-alkenyl), in the absence of exogenous acyl-CoA, increases with development (9), and this enhancement may be influenced by increasing myelin lipid.

Table 4 shows the amount of PA formed from DG by cortical gray matter homogenates. The specific activity of 18:1/18:1 DG kinase was significantly higher in newborns and 10-day-old rats than in 16-week-old and 26-week-old rats. The rate of phosphorylation of 18:1/18:1 DG seemed to decline with age during postnatal development. The active phosphorylation of 18:1/18:1 DG at the neonatal stage might have been induced by the enhanced synthesis of glycerolipid. However, the activity for 18:0/20:4 DG, which is a typical DG species produced from phosphatidylinositol, did not change. The different effects of age on the phosphorylation of 18:1/18:1 DG and 18:0/20:4 DG might be due to the existence of several DG kinase species in the brain. Lemaitre *et al.* (20) investigated the distribution of 20:4-specific and nonspecific isozymes of DG kinase in baboon tissues, and observed high activities of both isozymes in the brain. The results of this study suggest that the specific activity of glycerolipid synthesis in cortical gray matter declines as the rat cerebral cortex matures.

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TABLE 4
Phosphorylation of Diacylglycerol by Cortical Gray Matter Homogenates[a]

Diacylglycerol	0 d	10 d	30 d	16 wk	26 wk
			(nmol/min/mg of protein)		
18:1/18:1	2.67 ± 0.34[a]	2.34 ± 0.26[a]	2.15 ± 0.28[a,b]	1.81 ± 0.44[b]	1.78 ± 0.21[b]
18:0/20:4	1.62 ± 0.27	1.56 ± 0.16	1.65 ± 0.11	1.45 ± 0.44	1.39 ± 0.41

[a]Values (means ± SD of five litters or five rats) with different superscript letters (a,b) are significantly different ($P < 0.05$).

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Neutral Sterols of Sawflies (Symphyta): Their Relationship to Other Hymenoptera

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ABSTRACT: To investigate sterol utilization in sawflies, the neutral sterols of four species of sawflies were determined by gas–liquid chromatography and mass spectrometry, and compared to the respective dietary plant material. Cholesterol was the predominant (55–76%) sterol in all species and stages of sawflies examined. Host plants, however, contained primarily sitosterol (50–88%), along with other 24-alkylsterols and only 0.5–5.9% cholesterol, indicating that the sawflies examined are capable of dealkylating the C_{28} and C_{29} phytosterols in their diet to cholesterol. Comparative sterol metabolism in Hymenoptera is discussed.

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Insects require a dietary source of sterol for normal growth, development, and reproduction; a considerable amount of literature exists on the utilization and metabolism of dietary sterols by insects. It appears that although many plant-feeding insects are capable of converting dietary C_{28} and C_{29} phytosterols to cholesterol, other insects are incapable of this conversion, and their tissues contain little or no cholesterol (1–4). The orders Hemiptera (true bugs) and Hymenoptera (ant, bees, wasps) are interesting in this regard. Omnivorous or predaceous members of these taxa contain predominantly cholesterol, presumably because cholesterol is a major dietary sterol (5,6). However, other members of these groups that have developed phytophagy secondarily contain little cholesterol, and apparently do not possess the ability to dealkylate their dietary phytosterols. Included in this latter category are milkweed bugs (*Oncopeltus fasciatus*), cotton stainers (*Dysdercus fasciatus*), honey bees (*Apis mellifera*), several solitary bees, and certain phytophagous ants (7–13).

The exception to this pattern appears to be the Symphyta, a suborder of Hymenoptera generally referred to as "sawflies." All sawflies are primary plant-feeders, the only exception being the Orussidae, a single family that is predaceous or parasitic on wood-boring insects (14). Schaefer *et al.* (15) demonstrated that the Virginia pine sawfly *Neodiprion pratti* (Dyar) was able to dealkylate sitosterol, and three other species of sawflies, including *Dolerus nitens* Zaddach, *Xiphydria maculata* Say, and *Aneugmenus flavipes* (Norton), were shown to contain predominantly either cholesterol or 7-dehydrocholesterol, indicative of their ability to dealkylate plant sterols (16).

We have been able to acquire four additional sawflies species, including several of economic importance, and have compared the tissue sterols of these insects with their respective host plants in an effort to obtain the broadest picture of sterol utilization in this group of primitive phytophagous hymenopterans.

MATERIALS AND METHODS

Adults of *Macroxyela ferruginea* (Say) (f. Xyelidae) were collected on elm (*Ulmus* sp.) trees in Beltsville, Maryland. Adults of the red-headed pine sawfly *N. lecontei* (Fitch) (f. Diprionidae) were reared from larvae collected from pine (*Pinus* sp.) in Howard Co., Maryland, by trimming infested branches. Wheat stem sawfly *Cephus cinctus* Norton (f. Cephidae) adults were collected by sweep net from wheat near Three Forks, Montana, and adult *Atomocera decepta* Rohwer (f. Argidae) were reared from larvae collected in Beltsville, Maryland, on hibiscus (*Hibiscus moscheutos*). All insects and representative host plant samples were stored in MeOH at 4°C until workup. All laboratory analyses were done in triplicate; these analyses were conducted on pooled insect and pooled plant samples. Many of the insects only can be collected at specific times of the year, and then only for short periods. At these times, we collect as many insects as possible, then pool the sample. Therefore, we are basing our results, not on an individual or two, but rather a pooled population.

The MeOH from individual samples was decanted and reserved, and all samples were homogenized in $CHCl_3$/MeOH (2:1, vol/vol), filtered, and reextracted in fresh solvent. Combined filtrates, including the decanted methanol, were taken to dryness under vacuum. Residues were saponified by refluxing for 3 h using 6% KOH in a solution of EtOH/benzene/water (10:1:1, by vol). After cooling, the mixture was diluted with three volumes of water, acidified with 6 N HCl, and extracted three times with hexane. The hexane extracts were dried (overnight) over Na_2SO_4 and then taken to dryness under vacuum. The residues from all samples were fractionated on 60–100 mesh Florisil (5 g; Fisher Scientific, Fair Lawn, NJ) in a diethyl ether/hexane system as previously de-

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Abbreviation: GLC, gas–liquid chromatography.

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scribed (17). Neutral sterols were analyzed initially by capillary gas–liquid chromatography (GLC) at 245°C on a Shimadzu GC-9A gas chromatograph (Columbia, MD) equipped with a DB-1 fused silica column (15 m × 0.25 mm, 0.25 μm film; J&W Scientific, Folsom, CA), and identifications confirmed by GLC/mass spectrometry. Mass spectra were obtained on a Finnigan 4500 (Finnigan Corp., Sunnyvale, CA) fitted with a J&W DB-1 fused silica column (30 m × 0.32 mm, 0.25 μm film) and temperature programmed from 230 to 255°C (5°C increase/min). Electron impact data were collected at 70 eV and a source block temperature of 150°C.

RESULTS

The neutral sterols detected in the four species of sawflies examined along with the sterol composition of their dietary plant material were determined by GLC/mass spectrometry by comparison to known standards (summarized in Table 1). Cholesterol was the predominant sterol in all sawfly species and stages, ranging from over 55% in *N. lecontei* larvae to almost 76% in *M. ferruginea* adults. Other C_{27} sterols isolated from sawfly tissue included 7-dehydrocholesterol, desmosterol, lathosterol, and cholesta-5,22,24-trien-3β-ol. In contrast, the respective dietary plant material of these sawflies contained only relatively small amounts of cholesterol, ranging from 0.6% in pine needles to 5.9% in wheat stems. The predominant sterol in all host plants was sitosterol (50.2 to 83.5%), with lesser (and varying) amounts of stigmasterol, isofucosterol, and 24-methylcholesterol, depending on the particular plant species examined.

DISCUSSION

The high levels of cholesterol present in sawflies in this study, along with the relatively low proportions of cholesterol found in their respective dietary plant material, are indicative of an ability in these insects to dealkylate C_{28} and C_{29} phytosterols. The levels of desmosterol found in two of the sawfly species (*M. ferruginea* and *N. lecontei* adults) are also indicative of an ability to dealkylate, as desmosterol has been shown in radiolabeled studies to be an intermediate in the conversion to cholesterol by several insect species that dealkylate (1). Our present study, along with two previous studies (15,16), demonstrate that all eight species of sawflies examined to date can convert dietary phytosterols to cholesterol. Because these species represent four superfamilies and six families of the Symphyta, the ability to dealkylate is probably characteristic of the entire suborder, the one possible exception being members of the family Orussidae, which apparently have either a parasitic or predaceous association with wood-boring beetles (14).

The ability of sawflies to dealkylate phytosterols is different from phytophagous members belonging to the other suborder of Hymenoptera, the Apocrita comprising the bees, ants, and wasps. Ritter *et al.* (11) showed that brain and whole body extracts of the leaf-cutting ant *Atta cephalotes* (L.) contained only 24-methylene- and methylsterols and no cholesterol, indicating that the ant could not dealkylate the 24-methylsterols in its diet. Based on a comparison of dietary and tissue sterols, another fungus-feeding ant, *Acromyrmex octospinosus* (Reich), was also thought to be unable to dealkylate 24-alkylsterols (12). The honey bee *A. mellifera* was neither able to convert radiolabeled C_{28} and C_{29} sterols nor ^{14}C-desmosterol to cholesterol (10,18), and the solitary cactus bee *Diadasia rinconis* contained less than 0.1% cholesterol in its tissues (13). Interestingly, all of these plant (fungus or pollen)-feeding hymenopterans are secondarily phytophagous, having evolved from predaceous ancestors. Although strictly predaceous species of ants and wasps have been shown to contain primarily cholesterol (6), this may be more a function of a diet rich in cholesterol than an inherent ability to dealkylate C_{28} and C_{29} sterols. Examining orussids, the

TABLE 1
Relative Percentages of Major Neutral Sterols of Sawflies and Their Host Plants[a,b]

	Macroxyela ferriginea	Elm	*Neodiprion lecontei* (larvae)	*N. lecontei* (adults)	Pine	*Cephus cinctus*	Wheat	*Atomocera decepta*	Hibiscus
trans-22-Dehydrocholesterol[c]	—	—	—	—	—	—	—	10.1	—
Cholesterol	75.5	3.1	55.3	72.2	0.6	72.7	5.9	68.5	2.9
7-Dehydrocholesterol	—	—	11.7	—	—	1.6	—	0.9	—
Desmosterol	13.6	—	—	10.1	—	—	—	—	—
Lathosterol	0.6	—	—	—	—	—	—	—	—
Cholesta-5,22,24-trien-3β-ol	0.6	—	—	—	—	—	—	2.1	—
24-Methylcholesterol	1.5	4.6	3.5	2.0	8.2	14.6	22.4	6.8	10.8
Stigmasterol	—	1.1	—	—	0.7	0.7	11.4	0.7	31.0
Sitosterol	8.0	83.5	29.5	15.7	87.8	10.3	50.2	10.8	55.3
Isofucosterol	—	7.7	—	—	2.7	—	10.1	—	—

[a]Analysis of each compound by gas–liquid chromatography comparison to known standards, and verified by gas chromatography/mass spectrometry.
[b]Actual mass of sterol (mg per gram fresh weight): plants, 0.3–0.7; sawflies, 0.8–2.7.
[c]Trivial and systematic names: *trans*-22-dehydrocholesterol, cholest-5,22-dien-3β-ol; cholesterol, cholest-5-en-3β-ol; 7-dehydrocholesterol, cholesta-5,7-dien-3β-ol; desmosterol, cholesta-5,24-dien-3β-ol; lathosterol, 5α-cholest-7-en-3β-ol; 24-methylcholesterol, (24*R*)-24-methylcholest-5-en-3β-ol; stigmasterol, (24*S*)-24-ethylcholesta-5,22-*E*-dien-3β-ol; sitosterol (24*R*)-24-ethylcholest-5-en-3β-ol; isofucosterol, *trans*-24-ethylidene-cholest-5-en-3β-ol.

only nonphytophagous members of the Symphyta, might provide insight into comparative sterol utilization by members of the Hymenoptera.

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Identification of a Novel all-*cis*-5,9,12-Heptadecatrienoic Acid in the Cellular Slime Mold *Polysphondylium pallidum*[1]

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ABSTRACT: The all-*cis*-5,9,12-heptadecatrienoic acid was identified in the cellular slime mold *Polysphondylium pallidum*. The structural elucidation was accomplished by capillary gas chromatography, argentation thin-layer chromatography, and gas chromatography/mass spectrometry. This fatty acid has not been reported previously.
Lipids 31, 445–447 (1996).

Cellular slime molds provide an excellent model system for the study of cell-cell interactions. By starvation, the cells are triggered to enter a developmental cycle: over the course of a few hours, the cells begin to undergo chemotactic migration followed by the formation of multicellular aggregates.

Knowing the fatty acid composition is the first step in obtaining information with membrane fluidity in relation to cell-cell interactions. The fatty acid composition of the cellular slime mold *Dictyostelium discoideum* has been reported previously on both bacterially grown NC-4 and axenically grown Ax2 (1–3). Those compositions agreed well, and it was found that the total amount of unsaturated fatty acids was extremely high, comprising 75–90% of the total fatty acids of the organism and the membrane. Moreover, there was no clear difference between the fatty acid compositions prepared from the growth and aggregation phases in *D. discoideum*.

In the related species, *Polysphondylium pallidum*, there has been no previous report on the fatty acid composition. To determine the membrane components of the cells involved in cell-cell interaction, we have analyzed the fatty acids of *P. pallidum*.

We report here the occurrence of a novel fatty acid, all-*cis*-5,9,12-heptadecatrienoic acid, which, to our knowledge, has not been observed in nature before.

EXPERIMENTAL PROCEDURES

Polysphondylium pallidum strain WS320 was cultured as described previously (4). Total lipids were extracted from cells of the vegetative growth and aggregation competent phases by the method of Bligh and Dyer (5). The extracted lipids were subjected to methanolysis with 10% acetylchloride in methanol for 3 h at 90°C. The fatty acid methyl esters (FAME) were separated by thin-layer chromatography (TLC) on silica gel (E. Merck AG, Darmstadt, Germany) with hexane/diether ether/acetic acid (90:10:1, by vol) as solvent. After the plate was developed, it was dried and sprayed with a 0.01% primulin in a mixture of acetone and water (4:1, vol/vol). The spots on silica gel were detected under ultraviolet light, and one spot corresponding to FAME was recovered. The total FAME in the spot were extracted with hexane/methanol/500 mM NaCl in water (2:1:1, by vol), dried, and then redissolved in hexane. The total FAME were then separated by argentation TLC (AgTLC) on silica gel with hexane/diether ether (2:3, vol/vol) according to the degree of unsaturation. The FAME were analyzed with a Hitachi gas–liquid chromatograph (Model 263-30; Tokyo, Japan) equipped with a flame-ionization detector and capillary column (CPS-1; 50 m × 0.25 mm, i.d., 0.25 mm film; Quadrex, New Haven, CT). The separated FAME were identified by comparing their retention times with those of authentic standards as described previously (6). The positions of double bonds in the FAME were determined by the pyrrolidine method (7). For the further analysis of the positions of double bonds, trienoates were reduced partially with hydrazine and subjected to AgTLC with hexane and diether ether (80:20, vol/vol) as developing solvent. The monoenoate band corresponding to authentic methyl *cis*-monoenoates was scraped off and extracted with hexane. The purified monoenoate fraction was subjected to the I_2-catalyzed reaction for the formation of the adducts with dimethyl disulfide (DMDS) according to a minor modification (8) of the procedure of Shibahara *et al.* (9). Pyrrolidine derivatives and DMDS adducts were analyzed on an HP-5 column (30 m × 0.32 mm, i.d.; Hewlett-Packard, Palo Alto, CA) in a Hewlett-Packard HP-5890 gas chromatograph coupled to a JMS-AX500 (JEOL, Tokyo, Japan) mass spectrometer. The column temperature was programmed to increase

[1]Dedicated to Professors Tadao Yoshida and Masakazu Tatewaki on the occasion of their academic retirement.

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Abbreviations: DMDS, dimethyl disulfide; FAME, fatty acid methyl esters; TLC, thin-layer chromatography.

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from 260 to 300°C and from 230 to 280°C at a rate of 2°C/min for pyrrolidine derivatives and DMDS adducts, respectively.

RESULTS AND DISCUSSION

The FAME mixture was fractionated by AgTLC according to the degree of unsaturation. In the analysis on the AgTLC, we detected saturated, monoenoic, dienoic, and trienoic fatty acids in the total lipids of *P. pallidum* (data not shown). In the trienoate fraction, two peaks appeared in an analysis by gas–liquid chromatography (Fig. 1). Because no report on the presence of the trienoate in the cellular slime molds grown in nature exists to our knowledge, we tried to identify the peaks.

To ascertain the positions of double bonds in both of the peaks, a part of the trienoate fraction was subjected to pyrrolididation. Two peaks in the trienoate fraction were identified as heptadecatrienoic acid (peak 1) and octadecatrienoic acid (peak 2) as described later. Figure 2 shows the mass spectrum of the pyrrolidine corresponding to peak 1. These results indicate that the fatty acids, peaks 1 and 2, seem to be a novel 5,9,12-heptadecatrienoic acid and a 5,9,12-octadecatrienoic acid (data not shown), respectively.

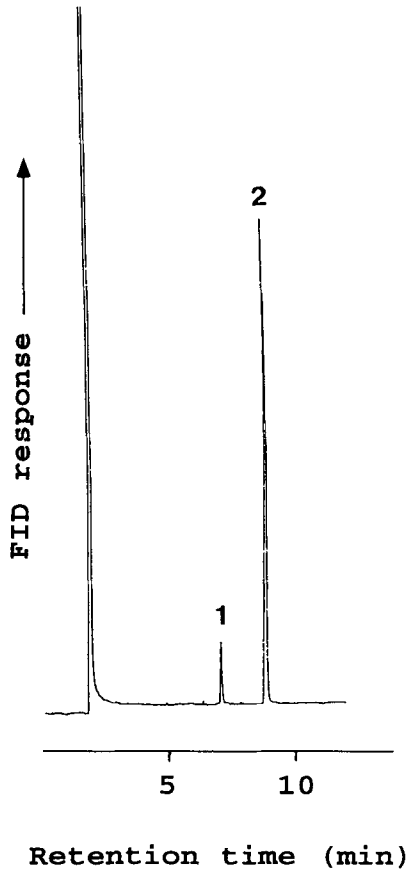


FIG. 1. Capillary gas chromatogram of fatty acid methyl esters of the trienoate fraction prepared from the aggregation-phase cells. Peak identifications are described in the text; FID, flame-ionization detector.

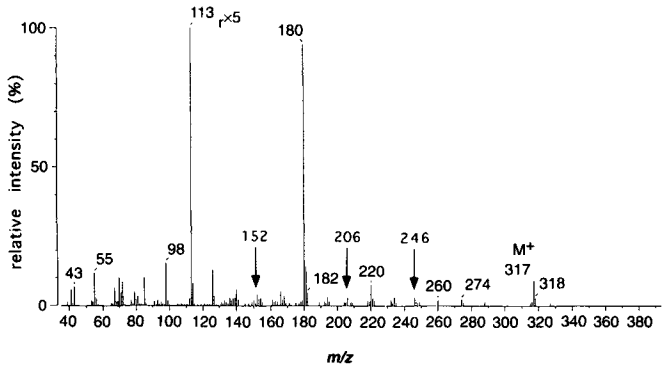


FIG. 2. Mass spectrum of heptadeca-5,9,12-enoylpyrrolidine. Arrows indicate the positions that differ by 12 mass units.

To confirm the results obtained, we used DMDS derivatives for analysis by gas chromatography/mass spectrometry. A part of the trienoate fraction was partially reduced with hydrazine; in the reduced products, a *cis*-monoenoate fraction was recovered by AgTLC, but no band corresponding to a *trans*-monoenoate was observed (data not shown). The DMDS adducts of the monoenoates were analyzed by gas chromatography/mass spectrometry. Figure 3 shows the mass spectra of the DMDS adducts recovered from the monoenoic FAME. The presence of DMDS adducts of 17:1(5), 17:1(9), and 17:1(12) methyl esters was confirmed, based on the detection of sets of the key fragmentation ions at *m/z* 215, 161, and 129 (Fig. 3A), at *m/z* 159, 217, and 185 (Fig. 3B), at *m/z* 227, 259, and 117 (Fig. 3C), respectively. The presence of the DMDS adducts of 18:1(5), 18:1(9), and 18:1(12) methyl esters was confirmed in the same manner by means of mass spectrometry (data not shown). The three types of 17:1 and 18:1 methyl esters recovered from the *cis*-monoenoate fraction were formed from 17:3 methyl ester and 18:3 methyl ester, respectively, by partial reduction with hydrazine, which is known to reduce double bonds without geometrical or positional isomerization of the remaining double bonds (8). Consequently, peaks 1 and 2 could be identified as all-*cis*-17:3(5,9,12) and all-*cis*-18:3(5,9,12), respectively; 18:3(5,9,12) has already been identified in the seed oil of a gymnosperm (10,11).

To the best of our knowledge, all-*cis*-17:3(5,9,12) identified here in *P. pallidum* has not been found previously in nature. The amount of trienoic fatty acids increased more in the aggregation competent phase than in the growth phase, concomitant with the decrease in the amounts of monoenoic and saturated fatty acids. This transition may provide the membrane with sufficient fluidity; under such conditions, the organism may be able to attain its morphogenesis at temperatures below 25°C. This heptadecatrienoic acid occupies about 1.5–2.0% of total fatty acids, whereas octadecatrienoic acid is a major component (17–27%) in the same acids. The composition analysis of the complete fatty acids should be undertaken as the next step of this study.

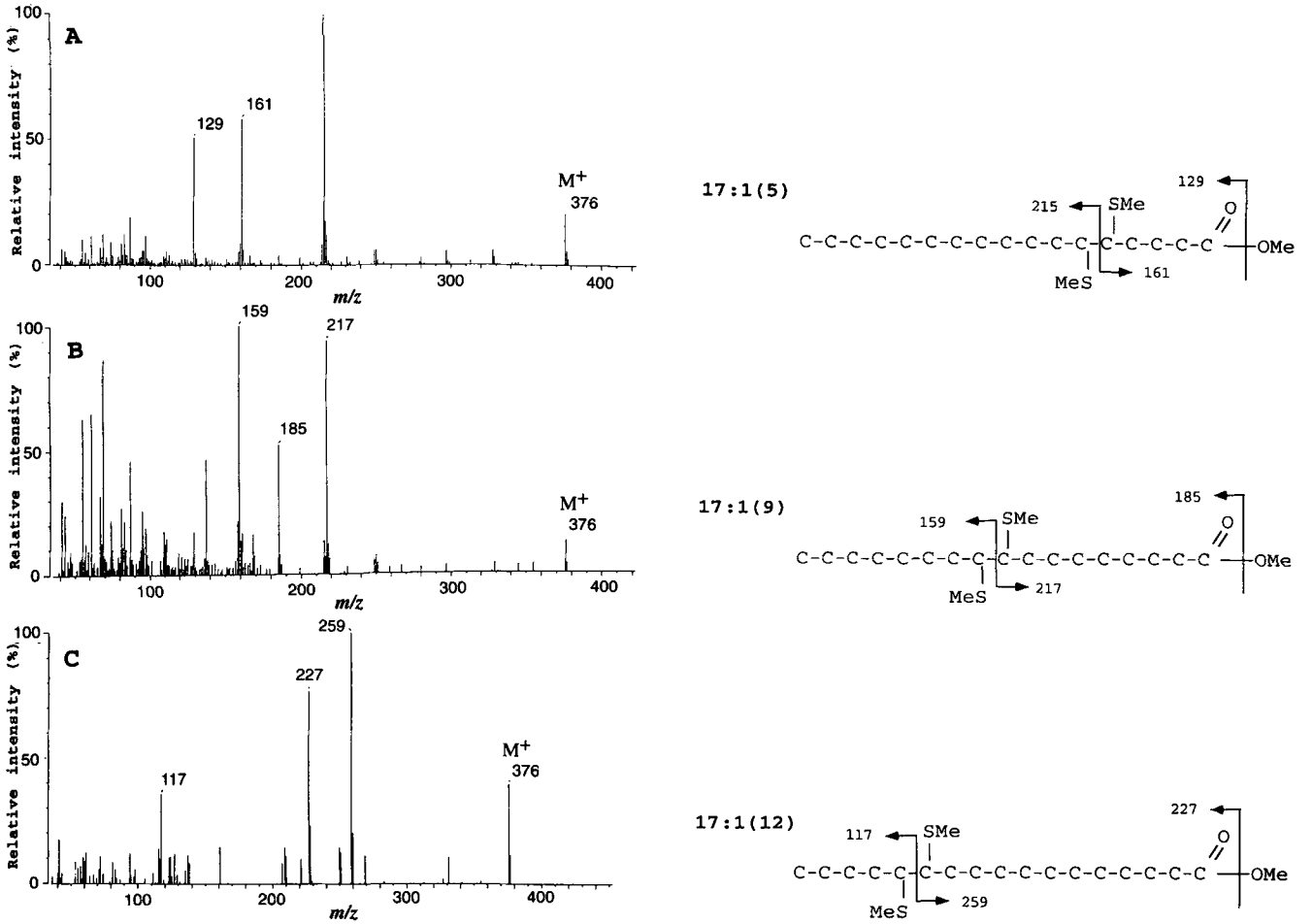


FIG. 3. Mass spectra of dimethyl disulfide adducts of total 17:1 methyl esters derived by partial hydrazine-reduction of the trienoate fraction. The schemes show fragmentation patterns of the individual dimethyl disulfide adducts of the methyl esters. Spectra A, B, and C are measured at different scan numbers on mass chromatograms.

ACKNOWLEDGMENTS

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ANNOUNCEMENTS

SPECIAL *LIPIDS* SUPPLEMENT—PROCEEDINGS OF THE ISSFAL 2ND INTERNATIONAL CONGRESS

Extra copies of the March *Lipids* Supplement—"Fatty Acids and Lipids from Cell Biology to Human Disease," the Proceedings of the 2nd International Congress of the ISSFAL (International Society for the Study of Fatty Acids and Lipids)—which includes 57 full manuscripts from participants in oral presentations at the Congress (332 total pages), are available for individual purchase at $60 each.

CALENDAR

May 1996

XVIth Washington International Spring Symposium on Frontiers in Bioactive Lipids—'96, May 6–9, 1996, Washington, D.C., USA. Information: Dr. Jack Y. Vanderhoek, Chair—Symposium '96, Dept. of Biochemistry and Molecular Biology, The George Washington University, 2300 Eye St., N.W. RN 5530, Washington DC 20037-2337 USA (Phone: 202-994-2929; Fax: 202-994-8974; E-mail: jyvdh@gwis2.circ.gwu.edu.). Deadline for submission of abstracts: February 1, 1996.

June 1996

Symposium on Diet, Nutrition and Health: An Update, University of Western Ontario from June 16–19, 1996, sponsored by the Royal Society of Canada. Sessions on Diet and Cardiovascular Disease, Diet and Cancer, Diet and Selected Health Problems, Including Hypertension, Diabetes, Obesity, Kidney Disease, Osteoporosis and Alcohol Abuse, as well as Diet and Health Maintenance in People at Various Stages of Life, Including the Elderly. Information: The Centre for Human Nutrition, The University of Western Ontario, London, ON N6A 5C1, Canada (Phone: 519-661-3096; Fax: 519-661-4006).

July 1996

European Thrombosis Research Organisation and the International Society for the Study of Fatty Acids and Lipids in cooperation with Summer University of Maastricht, July 10–12, 1996, University of Maastricht, The Netherlands, presents "Lipids, Membranes and Thrombosis (fundamental basis of cardiovascular disease and its prevention)." Information: Gerard Hornstra, Department of Human Biology, P.O. Box 616, 6200MD Maastricht, The Netherlands (Phone: +31-43-88 16 25; Fax: +31-43-67 09 76; E-mail: G. Hornstra @ HB.RuLimburg.NL).

September 1996

37th International Conference on the Biochemistry of Lipids, Antwerp, Belgium, Sept. 4–7, 1996. Themes: enzymes and transfer proteins related to lipoprotein metabolism, aspects of protein lipidation, and peroxisomal lipid metabolism. Poster communications may relate to the three conference themes or any other area of lipid metabolism. Information: Dr. P. van Dyck, Secretariat 37th ICBL, Department of Biochemistry, Physiology and Genetics, Division of Human Biochemistry, State University Centre Antwerp, Groenenborgerlaan 171, B2020 Antwerp, Belgium (Phone: 32 3 218 0329; Fax: 32 3 218 0326).

7th International Congress on Phospholipids, Sept. 9–11, 1996, Brussels, Belgium. Program topic areas include Growth, Death and Cancer; Choline, Choline Phospholipids and the Brain; Importance of Phospholipids in Health and Disease. Information: AOCS Meetings Department, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

IV. International Dermatology Symposion Berlin, Sebaceous Gland and Its Disorders, Basic and Clinical Research, Clinical Entities and Treatment, Sept. 13–15, 1996, University Medical Center Benjamin Franklin. Information: Docent Dr. Ch. C. Zouboulis, Department of Dermatology, University Medical Center Benjamin Franklin, The Free University of Berlin, Hindenburgdamm 20, D-12200 Berlin, Germany (Phone: 49-30-8445-2808; Fax: 49-30-8445-4262).

October 1996

Frontiers in Lipid and Lipoprotein Research: Basic Science, Analytical, Clinical, and Public Health Applications, Oct. 4–6, 1996, Stouffer Renaissance Hotel, Dallas, Texas. Sponsored by the Lipids and Lipoproteins Division of the

American Association for Clinical Chemistry, the Centers for Disease Control and Prevention, and the American Heart Association. An integrated review of relevant recent advances in basic science, measurement technology, epidemiological and clinical research, and public health issues will be presented. This review will help define state-of-the-art techniques for understanding and preventing morbidity and mortality due to heart disease. Information: Paula M. Steiner, Medical Research Laboratories, 2 Tesseneer Dr., Highland Heights, KY 41076 (Phone: 606-781-8877, Extension 252; Fax: 606-781-9310; E-mail: lipf@cehehll.em.cdc.gov).

Certification Board for Nutrition Specialists, Oct. 11, 1996, San Francisco, California. Certifying examination for nutritionists and dieticians with advanced degrees. Information: CBNS, 1562 First Avenue, Box #258, New York, NY 10028 (Phone: 212-332-0774 (leave message); Fax: 212-777-1103).

American College of Nutrition, 37th Annual Meeting, Oct. 11–13, 1996, San Francisco, California (Phone: 212-777-1037; Fax: 212-777-1103).

Seminar: Interconversion of Polyunsaturated Fatty Acids, and Their Functions in Membranes, Oslo, Norway, Oct. 11–12, 1996. Information: Georg Lambertsen, Secretary, Lipidforum, Breistølen 1, N-5035, Bergen, Norway (Phone: 4755 + 32 89 56; Fax: 47 55 + 32 57 24).

November 1996

AOCS Conference on PUFA in Infant Nutrition: Consensus and Controversies, Nov. 7–9, 1996, Barcelona, Spain. Information: Susan E. Carlson (E-mail: scarlson@utmem1.utmem.edu); Robert A. Gibson (E-mail: rgibson@paed_nnu.fmc.flinders.edu.au); Berthold Koletzko (Fax: 49-89-5160-4733); or AOCS Meetings Department, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

June 1997

The 19th Nordic Lipid Symposium, Ronneby, Sweden, June 15–18, 1997. Information: Georg Lambertsen, Secretary, Lipidforum, Breistølen 1, N-5035, Bergen, Norway (Phone: 47 55 + 32 89 56; Fax: 47 55 + 32 57 24).

July–August 1997

Fourth International Congress on Essential Fatty Acids and Eicosanoids, July 27–August 1, 1997, Edinburgh, Scotland. Information: Secretariat, c/o Meeting Makers, 50 George St., Glasgow G1 1QE, Scotland, United Kingdom.

ARTICLE

Phospholipid Composition of Neonatal Guinea Pig Liver and Plasma: Effect of Postnatal Food Restriction

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ABSTRACT: Preterm guinea pigs were delivered on day 65 of gestation (term = 68 d) and were allowed either free or restricted access to food for the subsequent 48 h. Plasma phosphatidylcholine (PC) concentration increased postnatally from 190 (range 144–307) to 751 (426–1039) and 883 (758–977) μM for fed and starved pups, respectively. Plasma PC composition in both groups of pups was characterized by selective and equivalent relative increases to individual molecular species containing 18:0 at the *sn*-1 position. Hepatic PC concentration increased from 6.75 (5.41–8.20) to 8.65 (6.54–10.63) and 9.23 (8.18–10.17) μmol/g for fed and starved pups, respectively, and, under all conditions, hepatic PC molecular composition closely mirrored that of plasma PC. These results support the hypothesis that the molecular species composition of plasma PC for the guinea pig in the immediate postnatal period is determined largely by the composition of the hepatic PC pool destined for lipoprotein secretion. Hepatic PC composition and concentration of the starved neonatal guinea pig were maintained independently of any dietary nutrient intake, at the expense of mobilization of extra hepatic lipid reserves. While this adaptive mechanism has inherent limited survival potential in neonatal starvation, it has implications for studies measuring plasma phospholipid fatty acid compositions as biochemical markers of dietary fat intake in preterm infants.

Lipids 31, 489–495 (1996).

Adequate supply of both n-3 and n-6 essential fatty acids (EFA) from the maternal circulation to the fetus and neonate is essential for optimal perinatal growth and development (1). A restricted capacity of fetal liver to desaturate and elongate the dietary EFA [linoleate (18:2n-6) and α-linoleneate (18:3n-3)] contributes to a requirement for the mother to supply long-chain polyunsaturated fatty acid (PUFA) products, arachidonate (20:4n-6) and docosahexaenoate (22:6n-3), respectively (2). The association between impaired neurological development and inadequate acquisition of 22:6n-3 in brain phospholipid has stimulated considerable research into the dietary PUFA content that is required to support optimal brain maturation, particularly in preterm infants (3,4). In general, these studies have concluded that improved neurological function in preterm infants can be achieved by balanced supplementation of infant formulas with 20:4n-6 and 22:6n-3 (5,6).

One major problem in human studies has been the lack of suitable biochemical markers to use in the evaluation of responses to changes in dietary formula fatty acid compositions. In this context, plasma phosphatidylcholine (PC) and erythrocyte PC and phosphatidylethanolamine (PE) fatty acid compositions have been regularly measured as surrogate markers of tissue phospholipid compositions (1,3–6). These measurements are not ideal, because plasma PC composition is dependent principally on the composition of hepatic PC (7), and erythrocyte phospholipid compositions are maintained by cell-specific acylation mechanisms (8). We have previously demonstrated, by analysis of the synthesis and secretion of hepatic and plasma PC molecular species in the rat (9) and guinea pig (10), that specificity of hepatic PC synthesis largely determines the composition of plasma PC. One major implication of this conclusion is that any dietary-associated changes to the composition of plasma PC must be mediated through corresponding adaptations to hepatic phospholipid metabolism.

In the present study, we have examined whether this hypothesis is supported by the changes to liver and plasma phospholipid composition that accompany the transition from placental to enteral nutrition at birth, using a neonatal guinea pig model. The guinea pig is in many respects an appropriate model to study perinatal changes to human infant lipid metabolism. In common with human infant development, the guinea pig exhibits a substantial accumulation of lipid in late gestation (11), its milk has a relatively low fat content (4%) (12), and it has a reduced capacity for ketone synthesis in the immediate postnatal period (13). In the present study, guinea pig pups were delivered 3 d preterm, and were either starved for 48 h or allowed to suckle freely for the same time period. Analysis of plasma PC and of hepatic PC and PE molecular species by high-performance liquid chromatography (HPLC) demostrated compositional and concentration changes that were substantially independent of postnatal dietary intake.

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Abbreviations: EFA, essential fatty acid; HPLC, high-performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid; TFE, trifluoroethanol.

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MATERIALS AND METHODS

Materials. HPLC grade methanol and trifluoroethanol (TFE) were obtained from Rathburn Ltd. (Walkerburn, Scotland). Chloroform and choline chloride were purchased from Merck Ltd. (Lutterworh, Leicestershire, United Kingdom). ATP, NADP, α-amylogycosidase (EC 3.2.1.3), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and hexokinase (EC 2.7.1.1) were purchased from Boehringer Mannhein UK (Lewes, East Sussex, United Kingdom). All other reagents and lipid standards were from Sigma (Poole, Dorset, United Kingdom).

Animal procedures. Female Dunkin-Hartley guinea pigs of known gestational age were obtained from our own colony as described previously (10,14). A total of twelve litters (32 pups) were delivered by Caesarian section, nine at day 65 gestation and three at day 67. Pups delivered on day 65 were randomly assigned to one control and two experimental groups, each containing animals from at least three litters. Pups in the control group were killed immediately at delivery, as were pups in a second control group which were delivered at day 67 of gestation. One group of pups delivered at day 65 was fed normally by being placed with lactating surrogate mothers for 48 h before being killed. The other group of pups delivered at day 65 were starved for 48 h by being placed with nonlactating adult female guinea pigs and allowed access to water, but not to solid food or edible bedding materials. Nonlactating dams were removed for 1 h each day and allowed free access to normal maintenance diet. All animals were killed by pentobarbitone injection. Whole-body weights were recorded for each pup at delivery, and also after 48 h for fed and starved animals. Blood was collected by cardiac puncture using EDTA as an anticoagulant. Plasma was separated from blood cells by centrifugation, and livers were removed and weighed rapidly before freezing in liquid nitrogen. All samples were stored at −20°C until analysis.

HPLC analysis of phospholipid molecular species. Liver PC and PE for HPLC analysis were prepared from 100-mg aliquots of liver tissue. Internal standards, 100 nmol PC14:0/14:0 in 25 μL TFE and 100 nmol PE14:0/14:0 in 100 μL chloroform were added, and homogenates in saline (0.8 mL) were extracted by chloroform/methanol as described previously (14). Plasma samples were extracted according to Bligh and Dyer (15) with the following modifications. Plasma (200 μL) was added dropwise with continual stirring to ice-cold methanol (2 mL) followed by addition of internal standard (20 nmol PC14:0/14:0 in 5 μL TFE), saline (600 μL), chloroform (2 mL), and water (1 mL). PC and PE were sequentially purified by solid-phase extraction using disposable BondElut aminopropyl cartridges (Phenomenex, Macclesfield, United Kingdom) (10,14); PC was eluted using chloroform/methanol (60:40, vol/vol) wash and PE using a subsequent methanol wash. Intact phospholipid molecular species were resolved by HPLC on a 3 μm, 25 cm × 4.6 mm APEX I ODS reverse-phase column (Jones Chromatography, Hengoed, Mid-Glamogan, UK) using a mobile phase of methanol/water (930:70, vol/vol) containing 40 mM choline chloride at a flow rate of 0.8 mL/min (16). Mass of eluted molecular species was quantified by postcolumn derivatization with 1',6-diphenyl-1,3,5-hexatriene and fluorescence detection (17). Individual molecular species were identified by retention time relative to the internal standard. Identities of species were further confirmed by reference to authentic standards, by the ratio of mass to ultraviolet absorption at 205 nm, and by gas-chromatographic analysis of fatty acid methyl esters derived from phospholipid species eluted from the HPLC (18).

Plasma cholesterol and triglyceride. Total plasma cholesterol and triglyceride concentrations were determined by cholesterol esterase (EC 3.1.1.13) or lipase (EC 3.1.1.3) hydrolysis followed by enzymatic/colorimetric assays linked to either cholesterol oxidase (EC 1.1.3.6) or glycerol phosphate oxidase (EC 1.1.3.21), respectively, using an RA2000 autoanalyzer (Bayer, Basingstoke, Hampshire, United Kingdom).

Liver glycogen. Liver glycogen from 100-mg aliquots of liver was determined as described by Keppler and Decker (19). Free glucose and total glucose, released by hydrolysis of glycogen in tissue homogenates by amyloglucosidase, were quantified separately in linked enzymatic assays (hexokinase/glucose-6-phosphate dehydrogenase) coupled to an increase in NADPH absorbance at 340 nm. The difference between the determinations represented the glycogen-specific glucose concentration.

Liver water. Liver water content was determined in accurately weighed (1 g) samples of liver which were lyophilized overnight in clean, dry, preweighed tubes, then oven-dried to constant weight.

Fecal lipid analysis. The fatty acid composition of total fecal lipid was determined by gas-chromatographic analysis of methyl fatty acids. Feces (1 g) were extracted using chloroform and methanol (2:1, vol/vol), saponified with methanolic KOH, and derivatized using boron trifluoride and methanol.

Statistical analysis. Statistical analysis was carried out using the Mann-Whitney U-test. All results are presented as median values, with ranges indicated.

RESULTS

Carcass and liver weights. The carcass and liver weights of animals delivered at day 65 and day 67 and killed immediately were essentially the same (Table 1). Similarly, these parameters were unchanged compared with birth weights for pups fed for 48 h after delivery. In contrast, starved animals showed a 20.7% fall in carcass weight compared with their birth weights, and a 24.7% decrease in liver weight compared with control pups delivered on day 65 (Table 1). The possibility that part of the fall in starved liver weight might have been due to dehydration was discounted by determination of liver water content in both fed and starved animals. There was no difference in the liver dry weights of starved or fed neonatal animals (Table 1). The possibility that starved animals may have received significant nutrition by coprophagic ingestion of fecal material from the surrogate mothers was addressed

TABLE 1
Neonatal Guinea Pig Carcass and Liver Weights[a]

	Day 65, killed at delivery (n = 9)	Day 67, killed at delivery (n = 9)	Pups fed for 48 h after delivery (n = 7)		Pups starved for 48 h after delivery (n = 7)	
			At delivery (day 65)	After delivery (48 h)	At delivery (day 65)	After delivery (48 h)
Body weight (g)	82.2 [74.7–97.8]	78.1 [70.1–95.2]	90.2 [77.9–104.6]	84.3 [78.2–100.8]	88.6 [67.1–119.3]	70.3 [57.0–90.6][b,c]
Liver wet weight (g)	3.59 [2.68–5.68]	3.48 [2.66–5.13]	ND	3.67 [3.10–5.00]	ND	2.62 [1.77–3.54]
Liver water (%)	62.3 [61.8–66.1]	61.7 [58.6–64.8]	ND	72.8 [71.4–76.8][b]	ND	71.1 [63.5–73.4][b]
Liver dry weight (g)	1.29 [1.01–2.07]	1.30 [1.02–2.10]	ND	0.98 [0.79–1.36][d]	ND	0.81 [0.50–0.94][d]

[a]Results of carcass weights, liver weights, and percentage liver water determinations in preterm guinea pigs. Values are expressed as medians with ranges; ND = not determined.
[b]Result significantly ($P < 0.05$) different from animals killed at delivery on day 65.
[c]Result significantly ($P < 0.05$) different from birth weight on day 65.
[d]Result significantly ($P < 0.0005$) different from animals killed at delivery on day 65. Values which were significantly different from animals killed on day 65 were also significantly different from values for animals killed at birth on day 67. All other comparisons between groups did not show a significant difference.

and largely discounted. There was no visually detectable material in the stomach lumens of starved animals. Moreover, fatty acid analysis of total lipid extracts of fecal material could detect only saturated and monounsturated fatty acids, principally palmitate, stearate, and oleate, and these were present at low concentration. Quantities of EFA, linoleate and linoleneate, and of their elongation and desaturation products were negligible.

Liver glycogen content. Liver glycogen concentration increased by 55% from 46.7 (17.7–52.9) mg/g wet wt on day 65 to 72.3 (41.2–105.3) mg/g wet wt on day 67 *in utero*. Liver glycogen in fed animals [44.2 (39.6–53.2) mg/g wet wt] was unchanged compared with the value at day 65, but was drastically reduced in starved animals to 2.0 (1.6–9.1) mg/g, $P < 0.001$.

Plasma PC and hepatic PC and PE concentrations. The concentration of PC in plasma increased slightly by 51% between day 65 and day 67 *in utero* ($P < 0.05$), but was subsequently more significantly elevated both in fed (4.0-fold, $P < 0.01$) and starved (4.6-fold, $P < 0.01$) neonatal guinea pigs (Table 2). Hepatic concentrations of PC (Table 3) and PE (Table 4) were the same for control newborn animals delivered on day 65 or day 67 of gestation. In contrast, hepatic PC concentration was elevated to a similar extent by 2 d of age in both fed (28.1%) and starved animals (36.7%) compared with values at birth for animals delivered on day 65 (Table 3). Liver PE concentration was increased only in fed (41.7%) animals (Table 4).

Plasma PC molecular species compositions. The seven molecular species detailed in Table 2 accounted for over 95% of total plasma PC. The distributions of plasma PC species were equivalent for newborn animals delivered on day 65 or day 67. The increased plasma PC total concentration between animals born on day 65 and day 67 was accounted for by general increased concentrations of all individual PC species, with the increases for PC16:0/22:6, PC16:0/20:4, PC18:0/22:6, and PC18:0/18:2 being significant at the 5% level. In contrast, the considerably greater elevated plasma

TABLE 2
Neonatal Guinea Pig Plasma Phosphatidylcholine (PC) Composition[a]

	PC (μM)			
Molecular species	Day 75, killed at delivery (n = 9)	Day 67, killed at delivery (n = 9)	Postnatal (48 h), fed (n = 7)	Postnatal (48 h), starved (n = 7)
PC 16:0/22:6	18 [13–28]	28 [19–49] (56)[b]	48 [30–58] (167)[c]	43 [29–56] (139)[c]
PC 16:0/20:4	9 [6–12]	17 [6–22] (89)[b]	28 [16–37] (211)[c]	36 [27–41] (300)[c]
PC 16:0/18:2	55 [41–83]	67 [47–115] (22)	129 [84–185] (135)[c]	167 [156–207] (204)[c]
PC 16:0/18:1	13 [7–22]	15 [12–31] (15)	30 [19–46] (131)[c]	37 [29–81] (185)[c]
PC 18:0/22:6	22 [9–26]	34 [24–64] (55)[c]	107 [57–150] (386)[c]	99 [67–143] (350)[c]
PC 18:0/20:4	14 [6–31]	23 [11–34] (64)	89 [43–123] (536)[c]	111 [87–139] (693)[c]
PC 18:0/18:2	70 [54–110]	110 [68–164] (57)[b]	319 [178–445] (356)[c]	363 [340–452] (419)[c]
Total	190 [144–307]	286 [197–456] (51)[b]	752 [426–1039] (296)[c]	883 [758–977] (365)[c]

[a]Results of high-performance liquid chromatography analyses of plasma PC molecular species compositions of preterm guinea pigs killed at birth, or delivered at 65 d gestation and either fed or starved for 48 h. Values are median concentrations with ranges and median percentage change compared with animals killed at delivery on day 65. There were no significant differences in plasma PC composition between animals either fed or starved for 48 h postdelivery. Values which were significantly different from animals killed at day 65 were also significantly different from newborn animals killed at day 67 ($P < 0.05$).
[b]Result significantly ($P < 0.05$) different from animals killed at delivery on day 65.
[c]Result significantly ($P < 0.01$) different from animals killed at delivery on day 65.

TABLE 3
Neonatal Guinea Pig Liver PC Composition[a]

Molecular species	PC (μmol/g) Day 65, killed at delivery (*n* = 9)	Day 67, killed at delivery (*n* = 9)	Postnatal (48 h), fed (*n* = 7)	Postnatal (48 h), starved (*n* = 7)
PC 16:0/22:6	0.72 [0.62–1.02]	0.66 [0.52–0.77] (–8)	0.86 [0.64–1.09] (19)	0.84 [0.69–0.94] (17)
PC 16:0/20:4	0.34 [0.29–0.58]	0.32 [0.25–0.39] (–9)	0.44 [0.33–0.51] (29)	0.48 [0.43–0.54] (41)[b]
PC 16:0/18:2	1.76 [1.29–2.14]	1.87 [1.47–2.27] (6)	1.57 [1.31–1.87] (–11)	1.54 [1.25–1.79] (–12)
PC 16:0/16:0	0.38 [0.20–0.57]	0.33 [0.20–0.50] (–13)	0.32 [0.22–0.46] (–16)	0.41 [0.34–0.49] (8)
PC 16:0/18:1	0.47 [0.30–0.77]	0.40 [0.3–0.53] (–15)[b]	0.65 [0.43–0.83] (38)	0.67 [0.63–0.81] (43)[c]
PC 18:0/22:6	0.77 [0.62–0.99]	0.85 [0.69–1.06] (10)	1.19 [0.79–1.55] (55)[c]	1.35 [0.92–1.47] (75)[c]
PC 18:0/20:4	0.43 [0.34–0.81]	0.38 [0.35–0.45] (–12)	0.69 [0.58–0.80] (60)[c]	0.80 [0.73–0.95] (86)[c]
PC 18:0/18:2	2.04 [1.51–2.17]	2.47 [1.98–2.83] (21)[c]	3.04 [1.94–3.67] (49)[b]	2.95 [2.64–3.44] (45)[c]
Total	6.75 [5.41–8.20]	7.29 [6.18–8.39] (8)	8.65 [6.54–10.63] (28)[b]	9.23 [8.18–10.17] (37)[c]

[a]Results of high-performance liquid chromatography analyses of hepatic PC molecular species compositions of preterm guinea pigs killed at birth, or delivered at 65 d gestation and either fed or starved for 48 h. Values are median concentrations with ranges and median percentage change compared with animals killed at delivery on day 65. See Table 2 for abbreviation. There was no significant difference in hepatic PC molecular species composition between fed and starved animals. Values which were significantly different for animals killed on day 65 were also significantly different from newborn animals killed on day 67 ($P < 0.05$).
[b]Values significantly ($P < 0.05$) different from animals killed at birth at 65 days gestation.
[c]Values significantly ($P < 0.01$) different from animals killed at birth at 65 days gestation.

PC concentration for both fed and starved postnatal animals was due to more significant increases in all PC species. The changes observed were essentially identical for both fed and starved animals. For both groups of postnatal animals, this increase in plasma PC concentration was more pronounced for species containing 18:0 rather than 16:0 at the *sn*-1 position. For fed and starved animals the mean percentage concentration increases for plasma *sn*-1 16:0 species were 161 and 207%, while corresponding values for *sn*-1 18:0 species were greater at 426 and 487%.

Hepatic PC molecular species compositions. For all groups of animals, the composition of hepatic PC (Table 3) closely resembled that of the corresponding plasma PC samples (Table 2), with the additional resolution of PC16:0/16:0. The eight major PC species reported (Table 3) consistently represented more than 88% of total hepatic PC. At delivery, the hepatic concentration of PC16:0/18:1 decreased slightly between day 65 and day 67, while that of PC18:0/18:2 increased (Table 3). In contrast, by comparison with compositions on day 65, the increased concentrations of liver PC in both fed and starved postnatal animals were characterized by more selective increased concentrations of individual molecular species. Strikingly, similar changes to hepatic PC composition were observed for both starved and fed neonatal guinea-pig pups (Table 3). In general, PUFA-containing PC species with 18:0 at the *sn*-1 position increased in concentra-

TABLE 4
Neonatal Guinea Pig Liver Phosphatidylethanolamine (PE) Composition[a]

Molecular species	PE (μmol/g) Day 65, killed at delivery (*n* = 9)	Day 67, killed at delivery (*n* = 9)	Postnatal (48 h), fed (*n* = 7)	Postnatal (48 h), starved (*n* = 7)
PE 16:0/22:6	1.34 [0.95–1.84]	1.03 [0.98–1.84] (–23)	1.73 [1.10–2.30] (29)	1.10 [0.81–1.74] (–18)[d]
PE 16:0/20:4	0.38 [0.19–0.82]	0.21 [0.11–0.39] (–45)	0.29 [0.14–0.35] (–24)	0.22 [0.16–0.27] (–42)[d]
PE 16:0/18:2	0.47 [0.27–0.67]	0.50 [0.31–0.58] (6)	0.54 [0.29–0.62] (14)	0.23 [0.11–0.36] (–51)[c,d]
PE 18:0/22:6	1.75 [1.21–2.19]	1.53 [1.10–1.99] (–13)	2.58 [1.74–2.98] (47)[c]	1.95 [1.79–3.73] (11)
PE 18:0/20:4	0.84 [0.55–1.27]	0.66 [0.45–0.83] (–21)[b]	1.18 [0.91–1.58] (40)	1.19 [0.88–1.74] (42)
PE 18:0/18:2	0.91 [0.73–1.66]	1.20 [0.54–1.44] (32)	1.69 [1.06–2.18] (86)[c]	1.46 [1.06–2.17] (60)[b]
Total	5.89 [4.06–8.07]	4.80 (4.28–6.68] (–19)	8.35 [5.29–9.39] (42)[b]	6.12 [4.96–9.77] (4)

[a]Results of high-performance liquid chromatography analyses of hepatic PE molecular species compositions of preterm guinea pigs killed at birth on day 65 or day 67 of gestation, and neonatal animals either fed or starved for 48 h after birth. Values are median concentrations with ranges and median percentage changes compared with pups killed at delivery on day 65. Values which were significantly different from animals killed on day 65 were also significantly different (at least $P < 0.05$) from newborn animals killed at day 67.
[b]Results were significantly ($P < 0.05$) different from animals killed at delivery on day 65.
[c]Results were significantly ($P < 0.01$) different from animals killed at delivery on day 65.
[d]Results were significantly ($P < 0.05$) different between animals fed or starved for 48 h after birth.

TABLE 5
Plasma Cholesterol and Triglyceride Concentrations[a]

	Concentration (mmol/L)			
	Day 65, killed at delivery ($n = 9$)	Day 67, killed at delivery ($n = 9$)	Postnatal (48 h), fed ($n = 7$)	Postnatal (48 h), starved ($n = 7$)
Cholesterol	0.8 [0.7–1.1]	1.1 [0.5–1.5]	2.4 [2.0–4.1][b]	3.0 [2.2–3.8][b]
Triglyceride	1.47 [0.88–1.79]	1.93 [0.52–2.79]	1.08 [0.86–1.68]	1.69 [1.15–5.28][c]

[a]Results of analyses of preterm guinea pig plasma cholesterol and triglyceride concentrations. Values are expressed as medians with ranges. Values which were significantly different from animals killed on day 65 were also significantly different from newborn animals killed on day 67.
[b]Result significantly ($P < 0.01$) different from animals killed at delivery on day 65.
[c]Result was significantly different between animals either fed or starved for 48 h after birth.

tion, while little change was observed for the corresponding species containing *sn*-1 16:0. The concentration increases for fed and starved pups were 54.5 and 75.3% for PC18:0/22:6, 60.5 and 86.0% for PC18:0/20:4 and 49.0 and 44.6% for PC18:0/18:2, respectively. There were smaller but still significant increases for PC16:0/20:4 (41.1%) and PC16:0/18:1 (42.5%) in livers from starved but not fed animals.

Hepatic PE molecular species compositions. Six major PE species are reported (Table 4) which comprise more than 95% of hepatic PE. The only differences in PE molecular species composition between pups delivered on day 65 and day 67 were decreased concentrations of the arachidonoyl-containing species PE16:0/20:4 and PE18:0/20:4 on day 67. As with both plasma and liver PC, the major changes observed by two days after delivery were similar for both fed and starved pups (Table 4). The concentration increases for fed and starved animals were 47.4 and 11.4% for PE18:0/22:6, 40.5 and 41.7% for PE18:0/20:4 and 85.7 and 60.4% for PE18:0/18:2, respectively. In contrast, for *sn*-1 16:0 PE species, the concentrations of PE16:0/20:4 and PE16:0/18:2 decreased by 23.7 and 42.1%, respectively, in livers from starved pups compared with corresponding values at delivery on day 65 (Table 4).

Plasma cholesterol and triglyceride concentration. Total plasma cholesterol and triglyceride concentrations (Table 5) showed no significant change *in utero* between day 65 and day 67. However, plasma cholesterol increased in fed (3.0-fold) and starved (3.8-fold) animals. Plasma triglyceride concentration was significantly elevated in the starved group (56.5%) compared with fed animals, but the starved animals also displayed a large range with one outlying lipemic value of 5.28 mmol/L.

DISCUSSION

The results from this study have demonstrated clearly that, in the preterm guinea pig, the postnatal changes to plasma PC composition observed in milk-fed pups were also apparent in pups subjected to acute, severe food restriction for 48 h. Not only was the overall increase in plasma PC concentration identical in fed and starved animals, but the detailed changes to PC molecular species compositions were also very similar (Table 2). Our observation agrees closely with studies on plasma phospholipid fatty acid concentration in human preterm infants who were either fed milk formula or who received lipid-free parenteral nutrition postnatally (20). By three days of age, total plasma phospholipid had almost doubled for both groups of infants, and no deficiency of long-chain PUFA was apparent for infants who did not receive any lipid nutrition.

We have previously demonstrated, by detailed metabolic studies of hepatic phospholipid metabolism in the rat (9) and guinea pig (10), that the composition of plasma PC is determined principally by the molecular specificity of hepatic PC synthesis. Comparison of the molecular species compositions of liver PC from stillborn infants and cord plasma PC suggested that a similar relationship holds for the human infant (21). In the present study, analysis of the molecular species compositions of plasma PC (Table 2) and liver PC (Table 3) was consistent with a similar mechanism acting in the postnatal guinea pig. For all experimental groups, the distribution of plasma PC species closely reflected that of the corresponding liver samples. There were relatively minor changes to the fractional compositions of both liver and plasma PC between day 65 and day 67 *in utero*, compared with the more significant and equivalent changes to both parameters for animals delivered on day 65 and killed 2 d later, which in turn were independent of the establishment of postnatal nutrition. The differential increases in both liver and plasma for PC species containing 18:0 at the *sn*-1 position were intriguing. We have previously reported that PC was synthesised *de novo* in perinatal guinea pig liver from CDP:choline principally as molecular species with *sn*-1 16:0, and *sn*-1 18:0 species were formed largely by acyl remodeling (10). The relative postnatal increase of *sn*-1 18:0 PC species in both liver and plasma could possibly be due to more rapid turnover of *sn*-1 16:0 species associated with increased acitivity of acyl remodeling pathways.

It is clear from the compositional analyses (Tables 3 and 4) that the major adaptations to hepatic phospholipid metabolism that occurred over the first two postnatal days were substantially independent of dietary input. Any contribution of coprophagic feeding by starved animals to these changes can be discounted because fecal material contained no n-6 or n-3 fatty acids that could have been precursors for the increased concentrations of polyunsaturated phospholipid species. The

similar extent of the increase in postnatal concentrations of plasma PC (Table 2) and plasma cholesterol (Table 5) suggests that a postnatal increased rate of hepatic lipoprotein secretion may also have been independent of food intake. These conclusions are supported by previous reports that, in other situations, the compositions of liver and exported lipoprotein phospholipid are sensitive to nondietary, hormonal influences. For example, the elevated 20:4n-6 content of hepatic PC in the female compared with the male rat is due principally to estrogen action (22), while specific increases of hepatic or plasma PC16:0/22:6 concentrations in human (21), rat (9), and guinea pig (14) at defined time points in gestation were independent of any change to the n-3 fatty acid intake of the maternal diet. While the hormonal factors regulating postnatal adaptations of hepatic phospholipid metabolism have not been investigated, our results are consistent with a previous implication of a central role for modification of plasma cortisol concentration (23).

Comparison of postnatal changes to circulating phospholipid composition and concentration in different animal species showed both similarities and significant differences. Total plasma phospholipid concentration increased in the initial days after birth for the preterm human infant (20,24) as well as for the guinea pig (Table 2). In contrast, the associated changes to plasma PC molecular species composition differed markedly between animal species and were possibly associated with the developmental timing of PUFA supply that is essential for brain maturation (25). For instance, assimilation of 22:6n-3 into brain phospholipid and neuronal maturation are initiated in mid-gestation for the human infant and continue for at least six months postnatally (26–28), but are both largely postnatal events for the rat (29). Consistent with the particularly high demand for PUFA to support human fetal brain development, fetal human plasma PC is selectively enriched in PUFA *in utero*, followed by a large increase in the content of 18:2n-6 after the establishment of enteral feeding (20,30). In contrast, the PUFA content of rat plasma PC increases only postnatally (31). Our analysis of plasma PC in the perinatal guinea pig, which showed no dramatic selective change to the content in PC species containing 22:6n-3 or 20:4n-6 compared with 18:2n-6 (Table 2), was compatible with previous reports that acquisition of 22:6n-3 into brain PE (18) and neuronal differentiation (32) are both essentially complete at term in this animal species.

Evidently, all EFA-containing phospholipid species in both the fetal and neonatal guinea pigs must have been derived from the maternal diet, either through placental transport or from milk. Presumably, mobilization of adipose tissue lipid reserves deposited *in utero* provided sufficient substrate to sustain postnatal changes to guinea pig plasma PC for at least two days in fasted animals, but obviously would be of limited use in the longer term. At the very least, however, it is clear from this response in starved guinea pig pups that the fatty acid composition of milk lipid cannot be the sole determinant of postnatal changes to plasma PC composition. A substantial contribution to such changes must come from adaptations to neonatal lipid metabolism that are essentially independent of postnatal dietary lipid input.

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Dietary Triacylglycerol Structure and Saturated Fat Alter Plasma and Tissue Fatty Acids in Piglets

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ABSTRACT: Human and pig milk triacylglycerols contain a large proportion of palmitic acid (16:0) which is predominately esterified in the 2-position. Other dietary fats contain variable amounts of 16:0, with unsaturated fatty acids predominantly esterified in the 2-position. These studies determined if the amount or position of 16:0 in dietary fat influences the composition or distribution of liver, adipose tissue, lung, or plasma fatty acids in developing piglets. Piglets were fed to 18 d with sow milk or formula with saturated fat from medium-chain triglyceride (MCT), coconut or palm oil, or synthesized triacylglycerols (synthesized to specifically direct 16:0 to the 2-position) with, in total fatty acids, 30.7, 4.3, 6.5, 27.0, and 29.6% 16:0, and in 2-position fatty acids, 55.3, 0.4, 1.3, 4.4, and 69.9% 16:0, respectively. The percentage of 16:0 in the 2-position of adipose fat from piglets fed sow milk, palm oil, and synthesized triacylglycerols were similar and higher than in piglets fed MCT or coconut oil. Thus, the amount, not the position, of dietary 16:0 determines piglet adipose tissue 16:0 content. The effects of the diets on the plasma and liver triacylglycerols were similar, with significantly lower 16:0 in total and 2-position fatty acids of the MCT and coconut oil groups, and significantly higher 16:0 in the plasma and liver triacylglycerol 2-position of piglets fed the synthesized triacylglycerols rather than sow milk or palm oil. The lung phospholipid total and 2-position 16:0 was significantly lower in the MCT, coconut, and palm oil groups, but similar in the synthesized triacylglycerol group and sow milk group. The lung phospholipid total and 2-position percentage of arachidonic acid (20:4n-6) was significantly lower in all of the formula-fed piglets than in milk-fed piglets. The physiological significance of this is not known.
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Intraluminal hydrolysis of dietary triacylglycerols by gastric and pancreatic lipase releases fatty acids from the 1 and 3 positions to form 2-monoacylglycerols and unesterified fatty acids, which are then absorbed and reassembled to triacylglycerols (1). Available estimates suggest that about 75% of fatty acids absorbed as 2-monoacylglycerols are conserved in the same 2-position as in the diet fat during this reassembly (2,3). Clearance of plasma triacylglycerols is mediated by lipoprotein lipase (LPL; EC 3.1.1.34) in the capillary beds. LPL activity increases dramatically in the rat and pig in the early postnatal period (4–6). This increase in LPL activity coincides with the rapid postnatal adipose tissue growth which occurs in many species, including pigs and humans. LPL can hydrolyze fatty acids from the 1- and 3-positions of triacylglycerols, but has restricted activity with fatty acids at the 2-position (7). Unesterified fatty acids released by LPL may be taken up by adipose tissue and re-esterified, or enter the circulation (8). Partial glyceride products may be further hydrolyzed by hepatic lipase, or possibly taken up by the liver in remnant lipoproteins. The 2-monoacylglycerol products could also be taken up by adipose tissue and reesterified *via* the monoacylglycerol pathway, or hydrolyzed by monoacylglycerol lipase (9).

The composition of human and pig milk, and the milk of several other animals is unusual in that palmitic acid (16:0) is preferentially esterified at the 2-position of the triacylglycerols (10,11). Pig adipose tissue, in contrast to humans and most other animals, also shows this unusual fatty acid distribution with the 2-position of the triacylglycerols largely occupied by 16:0 (12–15). Pig adipose tissue triacylglycerols, and pig and human milk fat, have been reported to have about 72–78, 58, and 55% 16:0, respectively, in fatty acids esterified at the 2-position (10,12,16). This specific positioning of 16:0 in the 2-position suggests that triacylglycerol synthesis in pig adipose tissue and human and pig mammary glands proceeds *via* the α-glycerophosphate pathway. Pig liver, in contrast, has a fatty acid distribution similar to other species with predominately oleic acid (18:1) and linoleic acid (18:2n-6) in the triacylglycerol 2-position (13). Recent studies have shown higher amounts of 16:0 in the 2-position of postprandial plasma triacylglycerols from breast-fed infants (17) and piglets fed sow milk (16) than in infants or piglets fed formula with 16:0 esterified to the triacylglycerol 1- or 3-positions. Whether the plasma triacylglycerol fatty acid distribution influences fatty acid delivery or distribution to adipose tissue, liver, or lung, for example, is unclear. The studies reported here determined if the distribution of fatty acids in milk or formula triacylglycerols influences the composition of fatty acids at the 2-position of adipose and liver triacylglycerols in piglets. Analysis of lung phospholipid fatty acids was included because of the possibility that 16:0 in the 2-po-

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Abbreviations: LPL, lipoprotein lipase; MCT, medium-chain triglyceride.

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sition of dietary fat could contribute to dipalmitoyl phosphatidylcholine in the growing lung.

METHODS

Animals and diets. Four ready-to-use liquid formulas were prepared by Mead Johnson (Evansville, IN) to contain similar levels of total saturated fatty acids, oleic acid (18:1), linoleic acid (18:2n-6), and linolenic acid (18:3n-3). Three formulas were prepared using high-oleic sunflower oil, sunflower oil and canola oil with saturated fatty acids added as medium-chain triglyceride (MCT) (8:0 plus 10:0), coconut oil (12:0 plus 14:0), or palm, mid-fraction (16:0). The formula containing MCT, and the formulas containing coconut or palm oil had a fat blend similar to that in some preterm and term infant formulas, respectively. The other formula contained synthesized triacylglycerols with the tradename Betapol^R. The synthesized triacylglycerols were made by interesterifying a tripalmitin-rich fraction of palm oil with sunflower and canola oils (16). The palm oil formula had 27.0% 16:0 with 4.4% 16:0 in the fatty acids esterified to the triacylglycerol 2-position, whereas the formula with synthesized triacylglycerols had 29.6% 16:0 with 69.9% 16:0 in the 2-position fatty acids (Table 1). The formulas were all low in cholesterol (<5 mg/dL) and devoid of carbon chain 20 and 22 n-6 and n-3 fatty acids. Complete details of the formula composition have been published (16).

Newborn, male piglets (Peter Hill Holdings, Langley, British Columbia, Canada), birthweight >1 kg were randomly assigned to the formulas and bottle-fed or left with the natural mother, six piglets per group. Littermates were not assigned to the same diet group. Passive immunity was provided to the bottle-fed piglets by addition of lyophilized immunoglobulins (La Belle Associates Inc., Bellingham, WA) to the formula for the first 72 h after birth (18).

At 18 d of age, and 10–12 h after the last feed, the piglets were anesthetized by intramuscular injection with ketamine/rompun (37.5/3.75 mg/kg; MTC Pharmaceuticals, Cambridge, Ontario, Bayvet Division, Chemagro Ltd., Etobicoke, Ontario, Canada). Fasting blood was used to allow study of predominately endogenous rather than absorbed triacylglycerols in plasma. Blood was drawn by cardiac puncture into tubes containing EDTA (150 mg EDTA/L in 9 g NaCl/L) as the anticoagulant, and plasma was prepared by centrifugation. Liver, abdominal subcutaneous tissue, and lung were quickly excised, blotted, weighed, homogenized, frozen in liquid nitrogen, then maintained at –80°C until analyzed. The procedures involving the piglets were approved by the Animal Care Committee of the University of British Columbia and conformed to the guidelines of the Canadian Council on Animal Care.

Biochemical analyses. Plasma and tissue total lipids were extracted, and the plasma and liver triacylglycerols and lung phospholipids were then separated from the other lipid classes by thin-layer plate chromatography (16,19). The composition of fatty acids at the 2-position of plasma, liver, and adipose tissue triacylglycerols and in the milk and formula fat as analyzed based on the methods of Kuksis (20) and Christie (21) as described elsewhere (16,17). Fatty acids were converted to their respective methyl esters using methanolic HCl (1:5 vol/vol) 100°C × 5 min for lung phospholipids and 14% boron trifluoride in methanol/benzene/methanol (25:20:25, by vol) 100°C × 30 min for triacylglycerols and 2-monoacylglycerols. Fatty acid methyl esters were separated using a Varian 3400 gas–liquid chromatograph equipped for analysis with capillary columns and quantitated using a Varian Star data system (Varian Canada Inc., Georgetown, Ontario, Canada) (17).

Statistical analysis. Results were compared among the groups of piglets fed formula and between each group of piglets fed formula and the group fed sow milk using analysis of variance. Formal tests for significant difference used Fisher's least significant difference and were done only for analysis of variance results with $P < 0.05$. The level of statistical significance was adjusted, using the Bonferroni correction,

TABLE 1
Fatty Acid Composition of Total Lipid and 2-Position Fatty Acids in Sow Milk and Formula Diets[a]

	Total fatty acids					2-Position fatty acids				
		Formula					Formula			
	Sow milk[b]	MCT[c] oil	Coconut oil	Palm oil	Synthesized TG[c]	Sow milk	MCT oil	Coconut oil	Palm oil	Synthesized TG
8:0	0.1	22.2	3.2	0.5	0.6	—	23.7	—	—	—
10:0	0.2	8.2	1.9	0.4	0.4	—	9.3	0.9	—	—
12:0	0.2	0.6	19.1	3.3	0.7	0.7	0.1	24.6	4.0	4.7
14:0	3.1	0.1	6.8	2.1	1.8	4.5	—	3.1	0.7	4.7
16:0	30.7	4.3	6.5	27.0	29.6	55.3	0.4	1.3	4.4	69.9
18:0	4.4	3.0	3.4	5.6	3.1	4.0	0.4	—	0.6	2.6
18:1	40.4	40.8	39.0	40.0	40.6	18.7	40.6	41.5	59.5	13.7
18:2n-6	8.2	16.2	15.6	16.4	16.4	4.9	22.3	23.2	25.1	6.5
18:3n-3	0.8	3.4	3.2	3.1	3.2	0.3	5.2	5.5	5.4	0.8

[a]The fatty acids are given as a percentage relative to the total fatty acids. Other details of the formula composition are in Innis and Dyer (Ref. 16).
[b]The sow milk also contained 1.1% carbon chain length 20 + 22n-6, including 0.7% 20:4n-6, and 0.7% carbon chain length 20 + 22n-3 fatty acids, including 0.2% 22:6n-3. The source of fat in the sows' diet was canola oil.
[c]MCT, medium-chain triglycerides; TG, triacylglycerols.

for the number of comparisons made, and was set at $P < 0.0125$ for comparisons among the groups fed formula and at $P < 0.008$ for comparisons between the groups fed formula and the group fed sow milk. All calculations were performed using the General Linear Models procedure in the Number Cruncher Statistical System, version 5.01 (Kaysville, Utah).

RESULTS

The sow milk and formula fatty acids all contained about 36–41% saturated fatty acids, but the levels of 8:0, 12:0, and 16:0, and the position of 16:0 in the triacylglycerols differed among the diets (Table 1). The formula with MCT had 22.2% 8:0 with 4.3% 16:0, and the formula with coconut oil 19.1% 12:0 with 6.5% 16:0. In contrast, the formula with palm oil or synthesized triacylglycerols, and the sow milk all had 27.0–30.7% 16:0. The sow milk and the formula with synthesized triacylglycerols had 55.3 and 69.9% 16:0, respectively, in fatty acids esterified at the 2-position. The formula with MCT, coconut and palm oil, however, had only 0.4–4.4% 16:0 in the 2-position fatty acids, and this was accompanied by higher levels of 18:1 (40–60% 2-position fatty acids), 18:2n-6 (22–25%), and 18:3n-3 (5.2–5.5%). The sow milk had 18.7% 18:1, 4.9% 18:2n-6, and 0.3% 18:3n-3; the formula with synthesized triacylglycerols had 13.7% 18:1, 6.5% 18:2n-6, and 0.8% 18:3n-3 in fatty acids esterified at the 2-position (Table 1).

The body weight of the piglets at 18 d of age was (mean ± SE) 5.1 ± 0.2, 4.4 ± 0.4, 4.3 ± 0.3, 4.4 ± 0.4, and 4.2 ± 0.4 kg for piglets fed sow milk, or the formula with MCT, coconut oil, palm oil, or the synthesized triacylglycerols, respectively. Body weight gain per liter of formula intake was not different among the four groups of formula-fed piglets: (mean ± SE) 293 ± 13, 292 ± 10, 274 ± 9, and 284 ± 3 g gain/L, respectively. The intake of sow milk could not be measured. The plasma cholesterol concentration of piglets fed sow milk was 4.47 ± 0.26 mmol/L and was significantly higher than the plasma cholesterol concentration of piglets fed the formula with MCT, 1.91 ± 0.10 mmol/L; coconut oil, 2.07 ± 0.10 mmol/L; palm oil, 2.17 ± 0.10 mmol/L; or synthesized triacylglycerols, 2.56 ± 0.18 mmol/L. The liver triacylglycerol concentration was significantly higher in piglets fed sow milk (mean ± SE 126 ± 3 μg/g liver) than in piglets fed the formula with palm oil (114 ± 2 μg/g) or synthesized triacylglycerols (103 ± 6 μg/g), but was not different from that of piglets fed the formula with MCT (123 ± 2 μg/g) or coconut oil (112 ± 7 μg/g). The liver triglyceride concentration of piglets fed the formula with MCT was also significantly higher than that of piglets fed the formula with palm oil or synthesized triacylglycerols.

The composition of the triacylglycerol total and 2-position fatty acids in the piglet plasma, liver, and adipose tissue, together with that of the diets, is shown in Figures 1 and 2. Arachidonic acid (20:4n-6) and 22:6n-3 were not present in the formulas, and because levels were low in all of the triacylglycerol fractions, data for these fatty acids are not included. The difference in the saturated fatty acid composition of the milk and formula diets resulted in similar differences in the saturated fatty acid composition of the piglet plasma, adipose tissue, and liver triacylglycerols. Piglets fed the formula with MCT or coconut oil, containing 8:0 or 12:0, respectively, as the major saturated fatty acid, had significantly lower levels of 16:0 in plasma, adipose tissue, and liver triacylglycerol total fatty acids than piglets fed sow milk or the formula with 16:0 from palm oil or synthesized triacylglycerols (Fig. 1). The fasting plasma triacylglycerol percentage of 16:0 was not significantly different between the piglets fed sow milk and those fed the formula with palm oil or synthesized triacylglycerols, although values for piglets fed the formula with palm oil were lower than values for piglets fed the formula with synthesized triacylglycerols. Similarly, the adipose tissue and liver triacylglycerol percentage of 16:0 was similar in piglets fed the formula with synthesized triacylglycerols and piglets fed sow milk, and significantly higher in both of these groups than in piglets fed the formula with palm oil. The higher percentage of 16:0 in the adipose tissue and liver of piglets fed milk or the formula with synthesized triacylglycerols could be due to improved absorption of 16:0 when present in the 2-position, rather than the 1 or 3-position of the dietary triacylglycerols (1,22,23). However, the slightly lower proportion of 16:0 in the palm oil than in the synthesized triacylglycerol formula or the sow milk (Table 1) could also explain these results. The significant differences in 12:0 and 14:0 in the piglet plasma and adipose tissue, and in 14:0 in liver among the groups (Fig. 1) may also be attributable to the differences in levels of these fatty acids in the diets. The very low amounts of 8:0, 10:0, and 12:0 in the liver triacylglycerols is probably explained by rapid, carnitine-independent oxidation and low activity of acyltransferases with medium-chain fatty acids (24,25).

Despite similar levels of 39–41% 18:1 in the milk and formula fatty acids, the differences in the formula saturated fatty acid compositions resulted in differences in 18:1, and to a smaller extent 18:2n-6 and 18:3n-3, in the piglet plasma, adipose tissue, and liver triacylglycerols. Levels of 18:1 and 18:2n-6 were significantly higher in liver, plasma, and adipose tissue triacylglycerols, and 18:3n-3 was significantly higher in plasma and adipose tissue of all piglets fed formula rather than sow milk. The higher 18:2n-6 and 18:3n-3 in piglets fed formula is probably explained in part by the higher formula content of 18:2n-6 and 18:3n-3 than that found in the milk (15.5–16.4% 18:2n-6 and 3.1–3.4% 18:3n-3 in the formulas and 8.2% 18:2n-6 and 0.8% 18:3n-3 in the sow milk). Piglets fed the formula with MCT oil had a significantly higher percentage of 18:1 in plasma, adipose tissue, and liver triacylglycerols than piglets fed the other formulas (Fig. 1). Levels of 18:1 in the plasma and liver triacylglycerols of piglets fed the formula with coconut oil were also significantly higher than those of piglets fed the formula with palm oil or synthesized triacylglycerols. Differences in 18:2n-6 and 18:3n-3 among piglets fed the different formulas were smaller, but generally followed a similar pattern to 18:1, with

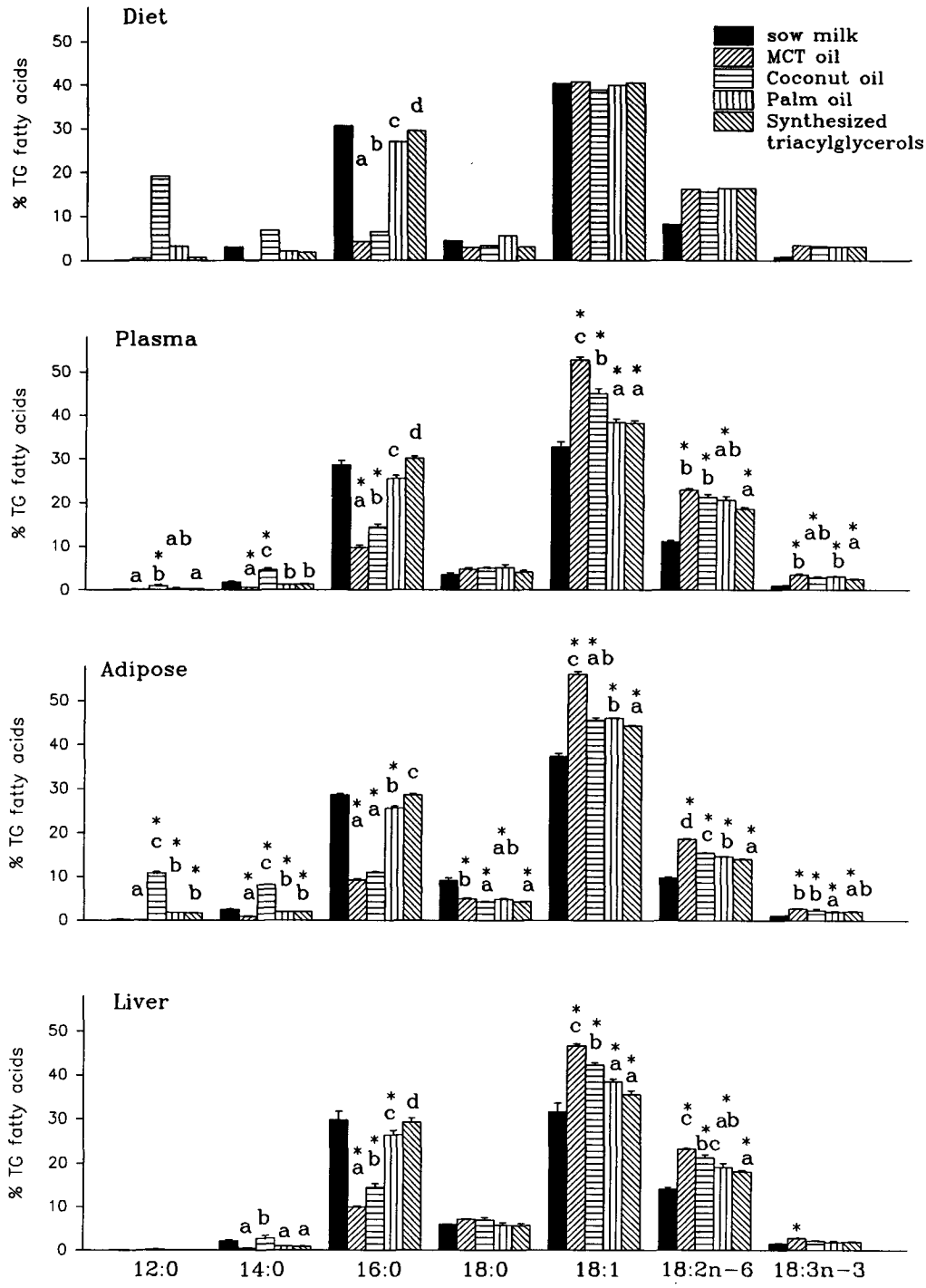


FIG. 1. Major fatty acid components (% total) of the diet, and of fasting plasma, adipose tissue, and liver triacylglycerols (TG) of piglets fed sow milk, or formula with medium-chain triglycerides (MCT) oil, coconut oil, palm oil, or synthesized TG from birth to 18 d of age. Values for plasma and tissue fatty acids are means ± SEM; *value significantly different from group fed sow milk, $P < 0.008$; ^{abcd}values for groups fed formula with a different letter are significantly different, $P < 0.0125$.

values for piglets fed the MCT > coconut oil > palm oil > synthesized triacylglycerol formula (Fig. 1).

The composition of fatty acids at the 2-position of the milk and formula fat also had a clear effect on the plasma, adipose tissue, and liver triacylglycerol 2-position fatty acids, with remarkably similar effects in the plasma and liver (Fig. 2). Piglets fed the formula with synthesized triacylglycerols had a significantly higher percentage, and piglets fed the formula

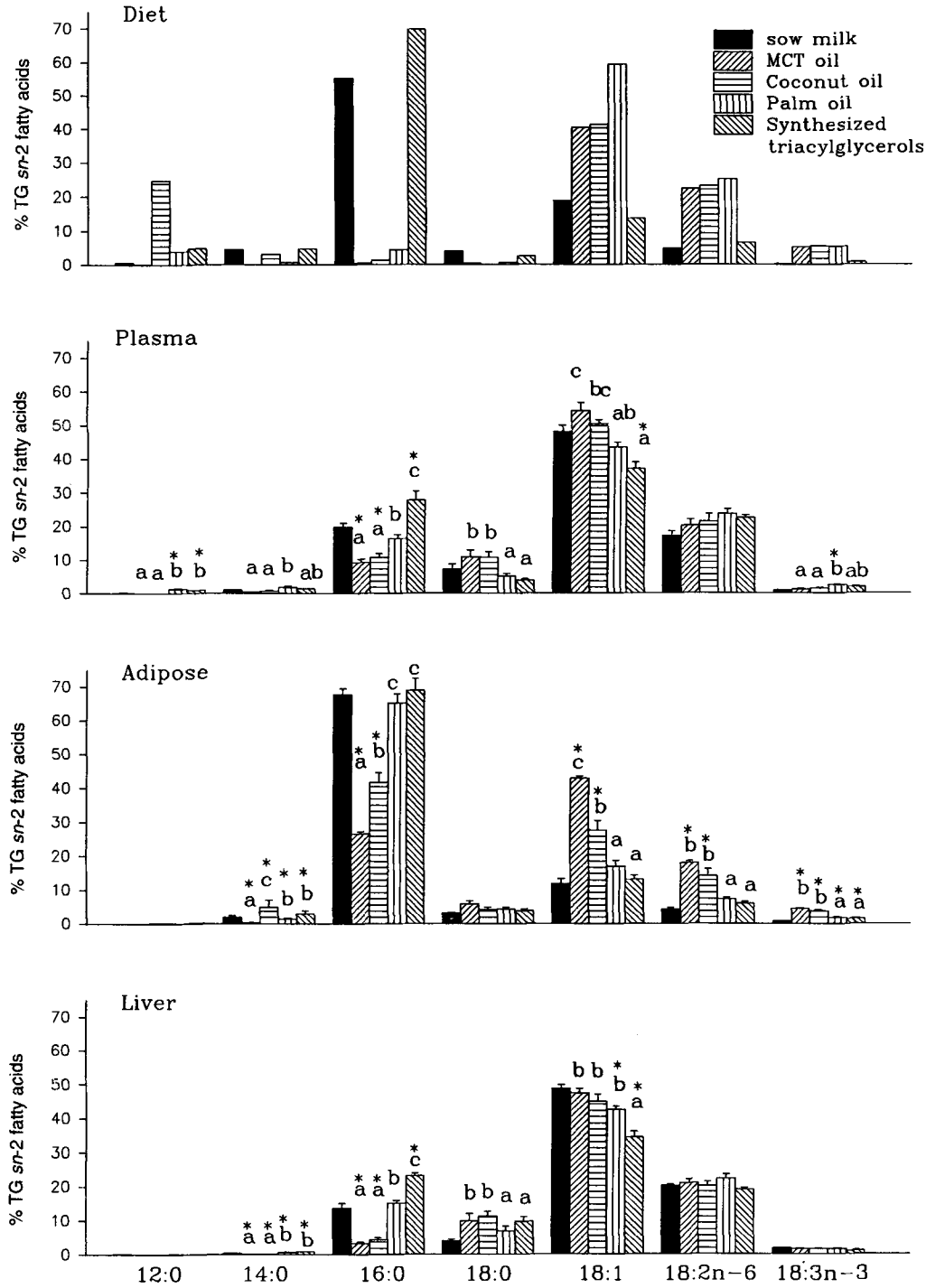


FIG. 2. Major fatty acid components (% total) esterified to the 2-position of the diet, and of fasting plasma, adipose tissue, and liver TG of piglets fed sow milk, or formula with MCT oil, coconut oil, palm oil, or synthesized triacylglycerols from birth to 18 d of age. Values for plasma and tissue fatty acids are means ± SEM; *values significantly different from group fed sow milk, $P < 0.008$; ^{abcd}values for groups fed formula with a different letter are significantly, $P < 0.0015$. Abbreviations as in Figure 1.

with MCT or coconut oil had a significantly lower percentage of 16:0 in plasma and liver triacylglycerol 2-position fatty acids than piglets fed sow milk or the formula with palm oil. In contrast, the percentage of 16:0 in the adipose tissue 2-position fatty acids was not different among piglets fed the formula with palm oil or synthesized triacylglycerols, and piglets fed sow milk.

The percentage of 18:1 in the 2-position of the plasma, adi-

pose tissue, and liver triacylglycerols showed a consistent pattern among the formula groups with piglets fed MCT > coconut oil > palm oil > synthesized triacylglycerols (Fig. 2). The differences among groups fed the MCT, coconut oil, and palm oil formulas are clearly not explained by differences in the percentage of 18:1 in the 2-position of the formula fat (Table 1). The percentage of 18:1 was significantly higher in the plasma and liver of piglets fed the formula with MCT, coconut oil, or palm oil, and in the adipose tissue of piglets fed the formula with MCT or coconut oil than in piglets fed the formula with synthesized triacylglycerols. The percentage of 18:1 in the 2-position fatty acids of adipose tissue from piglets fed the formula with MCT or coconut oil, in liver triacylglycerols from piglets fed the formula with palm oil, and in liver and plasma triacylglycerols from piglets fed the formula with synthesized triacylglycerols was lower than in piglets fed sow milk. Despite differences in the diets, there were no significant differences in the percentage of 18:2n-6 in the 2-position fatty acids of plasma or liver triacylglycerols, or in the percentage of 18:3n-3 in the liver triacylglycerols among piglets fed the different diets. In contrast, the percentages of 18:2n-6 and 18:3n-3 in the adipose tissue triacylglycerol 2-position fatty acids were significantly higher in piglets fed the formula with MCT or coconut oil than in piglets fed sow milk or the other formulas. The percentage of 18:3n-3, but not 18:2n-6, was also significantly higher in the adipose tissue of piglets fed the formula with palm oil or synthesized triacylglycerols than in piglets fed sow milk.

The major fatty acid components of lung phospholipid fatty acids, however, were significantly influenced by both the dietary saturated fatty acid composition and positional distribution (Table 2). Piglets fed the formula with MCT, coconut, or palm oil had significantly lower levels of 16:0 in lung phospholipid total and 2-position fatty acids than piglets fed sow milk. Values for piglets fed the formula with synthesized triacylglycerols, on the other hand, were not significantly different from that of the sow milk group. The percentages of 18:1 and 18:2n-6 in lung total and 2-position fatty acids were significantly higher, but 20:4n-6 was lower in all groups of piglets fed formula than in the group fed sow milk. The percentage of 20:4n-6 in total and 2-position fatty acids was also significantly lower in lung phospholipids from piglets fed the formula with MCT than in piglets fed the other formulas. No significant differences in lung phospholipid 18:2n-6 were found among the formula groups, or in 22:6n-3 among the milk or formula groups.

DISCUSSION

These studies show that the composition and distribution of dietary saturated fatty acids determine in part the composition and distribution of fatty acids in liver and adipose tissue, as well as in fasting plasma triacylglycerols of young piglets. The composition of adipose tissue fatty acids in piglets (26), pigs (13,15), human adults and infants, and other species (12,14,27–29) is known to correlate with the fatty acid composition of the diet. The 2-acyl group of adipose tissue triacylglycerols in most species, like that of plasma and other tissues, is usually an unsaturated fatty acid. The explanation for a specific positional distribution of adipose tissue fatty acids, other than a belief that lysophospholipid acyltransferase or remodeling reactions are specific for unsaturated fatty acids, is not clear. The adipose tissue from several species of the pig family, however, is unusual and different from that of humans and most other animals in that 16:0 is preferentially positioned at the 2 rather than the 1,3-positions (12–15). Thus un-

TABLE 2
Composition of Lung Phospholipid Total and 2-Position Fatty Acids from Piglets Fed Sow Milk or Formula[a]

Fatty acid	Sow milk	Formula			
		MCT oil	Coconut oil	Palm oil	Synthesized triacylglycerols
16:0 total	35.5 ± 0.8	25.2 ± 0.4[b]	25.8 ± 0.6[b,c]	31.3 ± 0.5[b,d]	34.2 ± 1.3[d]
16:0*sn*-2	25.9 ± 0.7	23.3 ± 0.8[b]	22.8 ± 1.0[b]	21.9 ± 0.2[b]	23.6 ± 0.7
18:0 total	11.2 ± 0.3	14.3 ± 0.3[b,e]	14.2 ± 0.2[b,d,e]	13.0 ± 0.2[b,d]	11.7 ± 0.3[c]
18:0 *sn*-2	5.9 ± 0.7	8.1 ± 0.6	6.0 ± 0.7	5.9 ± 0.6	6.5 ± 0.7
18:1	20.0 ± 0.4	29.6 ± 0.4[b,e]	26.7 ± 0.2[b,d]	23.5 ± 0.3[b,c]	22.8 ± 0.3[b,c]
18:1 *sn*-2	20.04 ± 0.4	26.0 ± 0.7[b,d]	24.6 ± 0.4[b,c,d]	23.3 ± 0.2[b,c]	22.8 ± 0.5[b,c]
18:2n-6 total	7.2 ± 0.3	11.2 ± 0.2[b]	11.3 ± 0.2[b]	10.9 ± 0.2[b]	10.8 ± 0.3[b]
18:2n-6 *sn*-2	7.9 ± 0.4	12.9 ± 0.5[b]	13.5 ± 0.3[b]	14.0 ± 0.5[b]	13.5 ± 0.5[b]
20:4n-6 total	10.5 ± 0.2	7.2 ± 0.2[b,c]	8.8 ± 0.1[b,d]	9.1 ± 0.2[b,d]	8.5 ± 0.3[b,d]
20:4n-6 *sn*-2	20.4 ± 0.9	13.9 ± 0.6[b,c]	17.1 ± 0.6[b,d]	19.1 ± 0.3[d]	17.5 ± 0.6[b,d]
22:5n-3 total	1.8 ± 0.1	1.1 ± 0.1[b,c]	1.3 ± 0.0[b,c,d]	1.5 ± 0.1[d]	1.4 ± 0.1[b,c,d]
22:5n-3 *sn*-2	3.2 ± 0.1	2.0 ± 0.0[b,c]	2.4 ± 0.1[b,c,d]	2.8 ± 0.1[d]	2.6 ± 0.2[b,d]
22:6n-3 total	0.8 ± 0.0	0.8 ± 0.0	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.0
22:6n-3 *sn*-2	1.4 ± 0.1	1.4 ± 0.1	1.6 ± 0.0	1.7 ± 0.1	1.6 ± 0.0

[a]Values are means ± SEM; *sn*-2 is used to indicate 2-position. Abbreviation as in Table 1.
[b]Values for group of formula-fed piglets significantly different from piglets fed sow milk, $P < 0.0125$.
[c,d,e]Values for piglets fed formula without a common superscript are significantly different, $P < 0.008$.

usual and specific positioning of 16:0 at the triacylglycerol 2-position is also a characteristic feature of human, pig, and several other mammalian milks (10,11,17). Again, the explanation for this is not clear.

The portal-venous transport of 8:0, 10:0, and 12:0, followed by rapid carnitine-independent oxidation in liver, and the low activity of acyltransferases with medium-chain fatty acids (24,25,30,31) are well known. Previous studies have suggested that preferential oxidation of 8:0–12:0 in animals fed diets with 8:0–12:0 rather than 16:0 as the source of saturated fat may result in higher availability of dietary 18:1 and 18:2n-6 for storage in growing tissue lipids (32). The results of this report, which show higher 18:1, and to a smaller extent higher 18:2n-6, in liver triglycerides of piglets fed the formula with MCT rather than oils with 16:0 as the source of saturated fat, are consistent with this.

The higher levels of 16:0 in the plasma and liver triacylglycerol 2-position fatty acids of piglets fed the formula with synthesized triacylglycerols than in piglets fed sow milk could be due to the higher percentage of 16:0 in the 2-position of the dietary synthesized triacylglycerols (69.9% 16:0) than in the sow milk (55.3% 16:0). However, the results of these studies also show significant amounts of 16:0 esterified at the 2-position of fasting plasma and liver triacylglycerols of piglets fed the formula with palm oil (mean ± SE percentage of 16:0, 16.4 ± 1.1 and 15.1 ± 0.8, for plasma and liver, respectively). Whether the high 16:0 in the 2-position of plasma triacylglycerols in piglets fed the palm-olein formula could have originated from monoacylglycerols with 16:0 released from adipose tissue is not known. Release of adipose tissue fatty acids involves hormone-sensitive lipase (EC 3.1.1.3) which hydrolyzes first one, then a second fatty acid from the triacylglycerol to form monoacylglycerol. Complete hydrolysis to glycerol and unesterified fatty acid is then catalyzed by monoacylglycerol lipase (EC 3.1.1.23) (33). Uptake and re-esterification of monoacylglycerols from adipose tissue and subsequent release in very low density lipoprotein triglycerides could explain the close relationship between the distribution of fatty acids in the piglet liver and plasma triacylglycerols found in this study. However, there seems to be no experimental evidence that monoacylglycerols are released by adipose tissues. However, consistent with the results of the study reported here, work by Christie and Moore (13) over 20 years ago noted about 36% 16:0 in the *sn*-2 position fatty acids of plasma triacylglycerols from domestic pigs, a substantially higher value than in the liver triacylglycerols.

The similar proportion of 16:0 in the 2-position of adipose tissue triacylglycerols in piglets fed sow milk and piglets fed the formula with palm oil or synthesized triacylglycerols, despite the large differences in the positional distribution of 16:0 in the diets, indicates that the α-glycerophosphate pathway probably predominates in adipose tissue triacylglycerol synthesis, at least in piglets. The much lower 16:0 in adipose tissue of piglets fed the formula with MCT or coconut oil, providing 8:0–12:0 rather than 16:0 as the major dietary saturated fatty acids suggests that exogenous fatty acids, rather than fatty acids synthesized *de novo*, are predominately used for the adipose tissue growth of young piglets. Similarly, others have shown that diets with safflower, sunflower, or canola oil, all of which are low in 16:0, decrease pig adipose tissue 16:0 when compared with a diet with animal fat (34). In the latter study, although the adipose tissue percentage of 16:0 was reduced by the vegetable oil diets, the proportion of total 16:0 in the 2-position was increased to 92%. Similarly, in the studies here, about 96% of the 16:0 in the adipose tissue triacylglycerols of piglets fed the formula with MCT or coconut oil was in the 2-position. Studies with rat adipose tissue have also suggested that, whereas the supply of exogenous fatty acids determines the relative proportions of fatty acids entering the triacylglycerols, the distribution of 16:0 and 18:1 to the 2-position is controlled entirely by the transacylases of the glycerol-3-phosphate pathway (14). Leray *et al.* (35), however, recently reported that the positional distribution of fatty acids in the white adipose tissue of rats fed fish oil was similar to that in the fish oil which was fed. Because the distribution of fatty acids in the 2-position of circulating triacylglycerols resembles that in the dietary fat (16,17,36,37), it is possible that 2-monoacylglycerols formed by LPL could be taken up and re-esterified *via* the monoacylglycerol pathway (9). Alternatively, esterification and remodeling reactions in fish and rats could be similar.

Several studies have suggested that the composition of lung phospholipid fatty acids, particularly 20:4n-6, is important to various lung functions (38–42). Studies on the effect of dietary fat on the lung have recently shown that the ability of lung to synthesize 20:4n-6-derived eicosanoids, which have many important roles in normal lung function, is reduced by dietary treatments which lower lung 20:4n-6 (40,41). The studies described here show that feeding formula, particularly formula low in 16:0, results in reduced lung 20:4n-6, when compared with milk feeding in piglets. Whether this has physiological significance, or if similar changes occur in infants fed formula, is not known. The results of these studies have also shown that the composition and distribution of saturated fatty acids in the dietary triacylglycerols influence the composition of fatty acids in the plasma, liver, and adipose tissue triacylglycerols of growing piglets. Whether or not the differences in liver fatty acid composition which result from feeding formula differing in saturated fatty acids, or which result from feeding formula rather than milk, are associated with any functional differences in this organ is not yet known. However, caution is warranted before extrapolating the results of these studies, particularly for adipose tissue, to the human infant fed formula because of the species differences in adipose tissue fatty acid distribution.

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Rat Gastric Hydrophobic Barrier: Modulation of Phosphatidylcholine Molecular Species by Dietary Lipids

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ABSTRACT: Phospholipids protect the gastric mucosa by forming a proton-repellent hydrophobic layer on its luminal surface. We have recently shown that two molecular species of phosphatidylcholine (PC), PC16:0/18:1, and PC16:0/18:2, but not PC16:0/16:0, are predominantly released into gastric mucus. We investigated whether these molecular species in mucus are modified by dietary fat. Rats were fed (for three weeks) a diet supplemented with either 10% cod liver, palm, or sunflower oil, or 10% corn starch as a control. In tissue, cod liver oil decreased PC16:0/20:4 and PC18:0/20:4. Cod liver oil and palm oil increased PC16:0/18:1, whereas sunflower oil decreased PC16:0/18:1. Palm oil additionally decreased PC16:0/18:2, whereas the other diets had no effect on PC16:0/18:2. In mucus, however, PC16:0/18:1 and PC16:0/18:2 were not significantly altered by any diet. They were increased over tissue values and comprised 37.6 ± 3.3 and 33.1 ± 1.4 mol% in controls. PC16:0/16:0 was lower in mucus than in mucosa and even decreased by cod liver oil (1.2 ± 0.2 vs. 2.7 ± 0.3 mol%; $P < 0.01$). We conclude that PC16:0/18:1 and PC16:0/18:2 are modified by dietary fat in tissue. In gastric secretions, however, PC16:0/18:1 and PC16:0/18:2 are kept constant and together comprise 70 mol% of the released PC species, whereas PC16:0/16:0 does not play a role for the gastric hydrophobic barrier under any dietary treatment. Additionally, cod liver oil decreases the content of PC16:0/20:4 and PC18:0/20:4 in gastric mucosa, thereby possibly decreasing the formation of eicosanoids.

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The gastric mucosa is normally able to resist the acidic environment of the stomach luminal contents, but a defective mucosal barrier against diffusion may result in the development of ulceral lesions (1–3). There is considerable evidence that the hydrophobic nature of gastric mucus is an essential factor in the protection of the gastric mucosal surface against the damaging effects of acid. Release of phosphatidylcholine (PC) by porcine gastric mucous cells is stimulated by prostaglandin E_2 (4), and the phospholipid content of gastric mucus is, in turn, thought to be a major determinant of its hydrophobicity (5,6). Consequently, by analogy with pulmonary surfactant, the term "gastric surfactant" has been used to describe gastric mucus phospholipids (7,8). However, any relationship between pulmonary surfactant and a putative gastric surfactant can only be superficial. We have recently demonstrated unambiguously that gastric mucus from both pig and rat contains only negligible amounts of PC16:0/16:0, the major surface tension-lowering component of lung surfactant. Instead, gastric mucus PC was enriched in the two unsaturated molecular species PC16:0/18:1 and PC16:0/18:2, suggesting a specific functional role for these two PC molecular species (9). One consequence of this suggestion could be that mechanisms exist to maintain a constant composition of gastric mucus PC that is substantially independent of dietary lipid intake. If so, then protective effects of dietary fats against mucosal damage, for instance that of n-3 fatty acids against ethanol-induced damage (10), cannot be mediated by changes of gastric mucus composition. In this study we have tested the hypothesis that composition of rat gastric mucus is not altered significantly by feeding diets based on palm oil, sunflower oil, or cod liver oil.

MATERIALS AND METHODS

Materials. Male rats (Wistar strain, 140–150 g body weight) were from the Zentralinstitut für Versuchstiere (Hannover, Germany). Disposable Varian 100 mg Bondelut^R NH_2-propyl columns were from Jones Chromatography (Hengoed, Mid Glamorgan, United Kingdom). Solvents for high-performance liquid chromatography (HPLC) were of HPLC grade from Baker (Deventer, The Netherlands). All other chemicals were of analytical grade and from various commercial sources.

Feeding experiments. Animals were given regular rodent chow for three days until start of the experiment. Afterwards, animals were fed for three weeks on a regular rodent chow supplemented with either 10% corn starch (controls), cod liver oil, palm oil, or sunflower oil (Ssniff Spezialdiäten GmbH, Soest, Germany). Fatty acid concentrations of the different diets are shown in Table 1. Food was withdrawn from the animals 20 h before sacrifice. The rats had free access to water at all times. Their body weights were 250–270 g after dietary treatment and did not differ between the experimental groups.

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Abbreviations: HPLC, high-performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

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TABLE 1
Amounts of Fatty Acids in Different Diets[a]

Fatty acid	Control	Cod liver oil	Sunflower oil	Palm oil
	(g • kg diet^{-1})			
14:0	n.d.[b]	4.9	0.1	1.1
16:0	5.0	18.2	13.5	48.0
16:1	0.1	10.2	0.2	0.3
18:0	0.8	3.2	5.4	5.2
18:1	6.4	34.4	30.7	45.9
18:2	15.7	27.1	77.6	27.1
18:3	1.8	3.9	2.4	2.3
20:1	0.2	10.7	0.1	0.2
20:5	n.d.	5.6	n.d.	n.d.
22:1	n.d.	6.3	n.d.	n.d.
22:5	n.d.	1.3	n.d.	n.d.
22:6	n.d.	4.1	n.d.	n.d.

[a]Diets were extracted with chloroform/methanol, the extracts transmethylated with acid methanol and fatty acid composition determined by gas chromatography. Fatty acid content was calculated from the content in total fat in the diet, i.e., 3% (w/w) in control diet and 13% (w/w) in the other diets.
[b]Not detected.

Analysis of fatty acids. Fatty acids of dietary fat were methylated/transmethylated according to Stoffel *et al.* (11). Methyl esters of fatty acids were separated on a fused silica CW20M-DF-0.25 column (17 m; Machery & Nagel, Düren, Germany) using a Varian (Palo Alto, CA) model 3400 gas chromatograph equipped with a flame-ionization detector. Peak area integration was performed by means of a Varian 4290 integrator.

Harvesting of gastric tissue and mucus. Gastric tissue and mucus fractions were harvested as previously described (9). Briefly, rats were injected intraperitoneally with 60 mg/kg body weight pentobarbital sodium (Nembutal®; Sanofi-Ceva, Luibourné, France) and 1500 I.U. heparin sodium (Liquemin®; Hoechst, Frankfurt/Main, Germany). After completion of anesthesia, the abdomen was opened and the stomachs were freed of blood by vascular perfusion with 150 mmol/L saline. The stomachs were excised and the lumen gently rinsed with 20 mL 150 mmol/L saline to remove any remaining food. The stomachs were then filled with 500 mmol/L saline at capacity and kept at 4°C for 20 min to harvest gastric secretions (8). Thereafter, the lumen of the stomach was vigorously lavaged with the 500 mmol/L saline three times (total volume 20 mL). The lavage fluid and post-lavage stomach tissue were stored at −20°C under nitrogen until use for lipid analysis.

Determination of PC molecular species. Phospholipids were extracted from gastric tissue according to the method of Folch *et al.* (12) and from gastric lavage fluid according to Bligh and Dyer (13). Phospholipids were quantitated according to Bartlett (14) and PC was prepared from lipid extracts by the following method, as described previously (9). In brief, 1 mg phospholipid was dissolved in 1 mL chloroform and placed on a 100-mg disposable NH_2-propyl Bondelut^R cartridge. After adsorption for five minutes the cartridge was washed twice with absolute chloroform to remove neutral lipids. No phospholipid was lost during this procedure. PC was eluted with 1 mL chloroform/methanol (70:30, vol/vol). Molecular species of PC were then separated according to Postle (15) by reverse-phase HPLC using methanol/water (925:75, vol/vol) supplemented with 40 mmol/L choline chloride as the mobile phase. Quantitation was achieved by fluorescence detection (excitation: 340 nm; emission: 460 nm) after post-column formation of mixed micelles with 1,6-diphenyl-1,3,5-hexatriene.

Statistics. One-way analysis of variance was calculated using GraphPad Instat Version 1.11a (GraphPad Software, San Diego, CA). Significance levels were corrected by means of the Bonferoni method for multiple group comparisons.

RESULTS

In gastric tissue PC16:0/18:1 and PC16:0/18:2 were the major PC molecular species comprising 15.5 ± 0.3 and 26.7 ± 0.7 mol%, respectively, in controls (Table 2). All dietary fats influenced the concentration of PC16:0/18:1. Cod liver oil and

TABLE 2
Effect of Diets on Phosphatidylcholine (PC) Molecular Species in Gastric Mucosa[a]

PC species	Control (n = 11)	Cod liver oil (n = 11)	Sunflower oil (n = 12)	Palm oil (n = 11)
PC16:0/16:0	7.0 ± 0.3	7.4 ± 0.3	6.9 ± 0.3	7.5 ± 0.2
PC16:0/18:1	15.5 ± 0.3	20.7 ± 0.2[b]	13.6 ± 0.2[b]	18.7 ± 0.2[b]
PC16:0/18:2	26.7 ± 0.7	27.4 ± 0.7	26.7 ± 0.8	22.9 ± 0.6[c]
PC16:0/18:3	2.3 ± 0.1	3.3 ± 0.4[d]	3.1 ± 0.1	1.7 ± 0.1
PC16:0/20:4	17.0 ± 0.5	10.0 ± 0.3[b]	14.5 ± 0.5[c]	16.9 ± 0.6
PC16:0/22:6	0.8 ± 0.1	2.2 ± 0.2	0.6 ± 0.1	0.6 ± 0.04
PC18:0/18:2	7.1 ± 0.3	8.1 ± 0.3	9.3 ± 0.3[c]	6.3 ± 0.2
PC18:0/20:4	11.0 ± 0.3	7.2 ± 0.3[b]	12.2 ± 0.3[d]	11.2 ± 0.2
PC18:1/18:2	4.5 ± 0.2	5.5 ± 0.1[c]	4.5 ± 0.2	4.9 ± 0.2
Others	8.1 ± 0.1	8.2 ± 0.3	8.6 ± 0.3	9.3 ± 0.2
Ratio: PC16:0/18:1 to PC 16:0/18:2	0.58 ± 0.02	0.76 ± 0.03[b]	0.51 ± 0.02	0.81 ± 0.03[b]

[a]Gastric tissue was extracted with chloroform/methanol, PC prepared from the extracts and PC species were separated by high-performance liquid chromatography as indicated in the Materials and Methods section.
[b]$P < 0.001$ vs. control.
[c]$P < 0.01$ vs. control.
[d]$P < 0.05$ vs. control.

palm oil increased PC16:0/18:1, but sunflower oil decreased PC16:0/18:1. PC16:0/18:2 was only decreased by palm oil. The ratio between PC16:0/18:1 and PC16:0/18:2 was increased by cod liver oil and palm oil for 31 ± 5 and 40 ± 5%, respectively ($P < 0.05$). Tissue PC16:0/16:0, however, was 7.0 ± 0.3 mol% in controls and remained unaffected by any diet. Highly unsaturated PC species, PC16:0/20:4 and PC18:0/20:4, were decreased by 41 ± 2 and 35 ± 3%, respectively ($P < 0.001$ each), by cod liver oil, whereas the other diets had no major effect. PC species containing 20:5n-3 were not detected in measurable amounts in gastric tissue.

Molecular species composition of mucus PC is shown in Table 3. Concentration of PC16:0/18:1 and PC16:0/18:2 was higher in mucus than in gastric tissue and comprised 37.6 ± 3.3 and 33.1 ± 1.4 mol% of total PC, respectively. In contrast to mucosal PC, no diet caused any significant change to these major components of mucus PC. There was only a tendency for the sunflower oil diet to increase mucus PC16:0/18:2 content at the expense of that of PC16:0/18:1; the ratio of PC16:0/18:1 to PC16:0/18:2 decreased from 1.15 ± 0.12 for animals on the control diet to 0.87 ± 0.04 for those on the sunflower oil diet (Table 3). Nevertheless, the PC16:0/18:1 to PC16:0/18:2 ratio was still higher in mucus than in gastric tissue of sunflower oil-fed rats. Concentration of the highly unsaturated PC species, PC16:0/20:4 and PC18:0/20:4, was less in mucus than in mucosa (Tables 2 and 3) ($P < 0.01$).

There were a number of changes in some of the minor components of mucus PC for animals fed experimental diets. Intriguingly, different effects were found depending on the nature of the fatty acid esterified at the *sn*-1 position, which would not have been apparent from simple fatty acid rather than molecular species analysis of PC. For instance, cod liver oil diet decreased mucus PC16:0/20:4 from 3.9 ± 0.5 to 1.9 ± 0.1 mol%, but had no effect on the content of PC18:0/20:4. And although concentrations of both 20:4n-6–containing PC species in mucus were increased by feeding rats the palm oil diet, this increase was considerably greater for PC18:0/20:4 (115%) than for PC16:0/20:4 (41%). The contents of neither PC16:0/20:4 nor PC18:0/20:4 were increased in gastric mucus in rats fed the sunflower oil diet, for which the only significant change was a selective increase in PC18:0/18:2 content from 8.0 ± 0.7 to 12.9 ± 1.5 mol%. The concentration of PC16:0/16:0, which comprised 2.7 ± 0.3 mol% of total mucus PC in rats fed the control diet, was not increased by any of the experimental diets. Instead, its content was decreased even further to 1.2 ± 0.2 mol% in rats fed the cod liver oil diet.

DISCUSSION

Although mucus phospholipids are thought to be an essential component for the hydrophobic protection of the gastric mucosa against acid (5–7), the molecular basis for this action remains unclear. It has been proposed that secreted gastric mucus PC represents a gastric surfactant analogous to the pulmonary surfactant layer lining the lung alveolus (7,16). Our previous findings demonstrated clearly, however, that this so-called gastric surfactant had a very different composition from pulmonary surfactant (9). PC16:0/16:0, the major lipid component of pulmonary surfactant, was present in only negligible concentration in gastric mucus. By contrast, PC16:0/18:1 and PC16:0/18:2 were the major PC molecular species released into gastric mucus; moreover, these two species were significantly enriched in gastric mucus compared with mucosal PC.

The present study provides confirmatory evidence for our hypothesis that this composition of mucus PC secretion from the gastric mucosa is tightly regulated. Modification of the composition of dietary fat produced relatively substantial changes in mucosal PC composition, but exerted a much less significant effect on the PC composition of the mucus fraction. For all three experimental diets, irrespective of changes to gastric tissue PC, the sum of PC16:0/18:1 and PC16:0/18:2 as the major PC species was consistently higher in mucus

TABLE 3
Effect of Diets on PC Molecular Species in Gastric Mucus[a]

PC species	Control ($n = 6$)	Cod liver oil ($n = 9$)	Sunflower oil ($n = 6$)	Palm oil ($n = 6$)
PC16:0/16:0	2.7 ± 0.3	1.2 ± 0.2[b]	2.2 ± 0.2	1.4 ± 0.2
PC16:0/18:1	37.6 ± 3.3	40.8 ± 0.8	33.1 ± 0.9	39.0 ± 1.1
PC16:0/18:2	33.1 ± 1.4	32.5 ± 1.4	38.2 ± 0.4	28.1 ± 1.5
PC16:0/18:3	1.6 ± 0.7	1.6 ± 0.2	2.1 ± 0.3	1.8 ± 0.3
PC16:0/20:4	3.9 ± 0.5	1.9 ± 0.1[b]	3.4 ± 0.4	5.5 ± 0.3[c]
PC16:0/22:6	0.2 ± 0.3	0.6 ± 0.2	0.0 ± 0.0	0.1 ± 0.1
PC18:0/18:2	8.0 ± 0.7	7.3 ± 0.2	12.9 ± 1.5[c]	7.3 ± 0.6
PC18:0/20:4	3.9 ± 1.1	5.5 ± 0.5	2.3 ± 0.4	8.4 ± 1.0[c]
PC18:1/18:2	2.8 ± 0.8	1.7 ± 0.3	1.3 ± 0.3	2.0 ± 0.2
Others	6.7 ± 1.6	6.8 ± 0.5	4.5 ± 0.9	6.3 ± 0.8
Ratio: PC16:0/18:1 to PC 16:0/18:2	1.15 ± 0.12	1.26 ± 0.04	0.87 ± 0.04[c]	1.40 ± 0.08

[a]Mucus was extracted with chloroform/methanol, phosphatidylcholine (PC) prepared and separated with high-performance liquid chromatography as described in the Materials and Methods section.
[b]$P < 0.01$ vs. control.
[c]$P < 0.05$ vs. control.

than in mucosa. This difference was probably an underestimate, as the mucosal fraction will have contained a potentially substantial, but not readily quantifiable, contribution from nascent mucus PC. While cod liver oil and palm oil diet significantly increased the ratios of PC16:0/18:1 to PC16:0/18:2 in gastric tissue (Table 2), they exerted a much lower effect on mucus PC (Table 3). Importantly, none of the experimental diets increased the low content of PC16:0/16:0 previously reported in both gastric mucosa and mucus of rats fed standard control diet (9). This result confirmed that modification of PC16:0/16:0 secretion into gastric mucus PC does not contribute significantly to any hydrophobic protection of the gastric mucosa.

Identification of the effective absense of PC16:0/16:0 in gastric mucus PC has far reaching consequences for our understanding of the mechanism underlying the protection of the gastric mucosal surface by hydrophobic components, in many respects simplifying the concepts involved. PC16:0/16:0 is essential in pulmonary surfactant to oppose surface tension forces at the air–liquid interface in the lungs (17). This function is dependent on the high gel to sol phase transition temperature (41°C) of PC16:0/16:0 which, consequently, is an inert solid material at body temperature (18). Pulmonary surfactant contains specialized unsaturated phosphatidylglycerol and specific apoprotein components designed to facilitate adsorption of this solid PC16:0/16:0 to the air–liquid interface (17,18). These constraints imposed on pulmonary surfactant by the saturated nature of PC16:0/16:0 will not apply to gastric surfactant, as this is composed principally of the more fluid molecules PC16:0/18:1 and PC16:0/18:2. This conjecture is in agreement with our previous findings that negligible PG was present in gastric secretions of either pig or rat (9). One protein characteristic of lung surfactant, surfactant protein D, is expressed by gastric mucosal cells (19), but this protein is not thought to contribute directly to the surface activity. Rather, it is a component of the innate defense system and probably contributes in both tissues to host defense rather than to surfactant function (17,19).

The lack of effect of cod liver oil diet on the PC molecular species composition of gastric mucus demonstrated clearly that its protective action against ethanol-induced tissue damage (20) was not due to modification of any hydrophobic protective mechanism. Instead, feeding cod liver oil dramatically decreased concentrations of arachidonoyl-containing species PC16:0/20:4 and PC18:0/20:4 in gastric mucosa, resulting in a decrease of potential substrate for generation of noxious leukotrienes C_4 and D_4 (10,20) and leaving unaffected or even improving the vascular perfusion of gastric mucosa (21,22). Consequently, any protective effect of n-3 fatty acids on the gastric mucosa may be due to modulation of release of such arachidonic acid-derived eicosanoid mediators.

Although in gastric mucus PC comprises 60 mol% of total phospholipids, the concentration of phosphatidylethanolamine (PE) is only 20 and 30 mol% in the rat and in the pig, respectively (9). Although *de novo* synthesis rates of PC and PE are similar in isolated porcine gastric mucous cells, palmitic acid incorporation into PE is only 15–20% the value of PC (23). These findings show that palmitoylated PE species do not play a major role in gastric secretions. However, a contribution of PE to the hydrophobic barrier of the stomach cannot be discounted. So far, no data exist for the gastric hydrophobic barrier in humans, particularly not in terms of individual molecular species of phospholipids. Therefore, further studies will be necessary to investigate the phospholipid concentration and composition as well as the regulation of phospholipid synthesis and secretion in the stomach in humans. This is particularly important as impaired hydrophobicity facilitates the development of gastric lesions in humans (5,24–26).

We conclude that PC16:0/18:1 and PC16:0/18:2, but not PC16:0/16:0, are the major PC species being released from the mucosa into gastric mucus. The concentration of these PC molecular species is kept relatively constant in mucus. Therefore, the molecular basis of the hydrophobic barrier of the stomach is different from that of the pulmonary surfactant. We further conclude that protection of the stomach by dietary n-3 fatty acids may be mediated by decreasing arachidonic acid, thereby leading to less formation of noxious leukotrienes, rather than by influencing the molecular composition of the gastric hydrophobic barrier.

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Changes in Lipid Composition During Metamorphosis of Bonefish (*Albula* sp.) Leptocephali

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ABSTRACT: During metamorphosis of bonefish (*Albula* sp.) larvae (leptocephali) all energy requirements are provided by breakdown of endogenous compounds, with lipid catabolism accounting for about 80% of total energy production. The principal objective of the present study was to characterize the lipid classes and fatty acids utilized. Analysis of whole-body lipid content indicated that larvae lost about half (3.6 mg) of their total lipid during the 10-d period. Percentages of neutral and polar lipid in early metamorphosing larvae were 64.2 and 35.8%, respectively; these values showed little change during metamorphosis, indicating that both lipid classes were catabolized. Triacylglycerols, the principal neutral lipid of all metamorphic stages, decreased by 1.8 mg, accounting for half of the decrease in total lipid. Levels of phosphatidylethanolamine, the principal polar lipid in early larvae, decreased by more than 50% during metamorphosis; levels of phosphatidylcholine, which was not detected in early larvae, increased. Fatty acids showing the largest net decreases, presumedly used as energy sources, were 16:0 (30.4%), 14:0 (13.8%), 16:1n-7 (12.2%), 20:5n-3 (7.7%), 18:1n-9 (7.4%), and 18:4n-3 (6.9%). Most of 22:6n-3, the second most abundant fatty acid of early larvae, was conserved.

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Metamorphosis of leptocephalous larvae of the bonefish from the Gulf of California (Albuliformes: Albulidae: *Albula* sp.) is characterized by rapid and dramatic changes in morphology and chemical composition (1,2). During a period of approximately 10 d, metamorphosing leptocephali lose about 80% of their wet mass and about 60% of their length as they transform into juvenile fish (3). Nutrient requirements are provided by catabolism of endogenous reserves formed during the premetamorphic growth interval and are stored mainly in the transparent, extracellular gelatinous matrix that forms the bulk of the body mass (4); feeding does not resume until near the end of metamorphosis (5; Pfeiler, E., unpublished data).

Previous studies on changes in biochemical composition indicated that endogenous substances used as energy sources are lipid and carbohydrate (keratan sulfate glycosaminoglycan) (2). Metamorphosing larvae show about a 50% net loss of total lipid and about an 80% loss in keratan sulfate. Lipid breakdown accounts for about 80% of the total energy requirements during metamorphosis (Pfeiler, E., unpublished data).

Given the importance of lipid as a nutrient source during this period of rapid and extensive remodeling of larval tissues, we addressed the following questions: (i) What is the lipid class and fatty acid composition of bonefish leptocephali at the beginning of metamorphosis? (ii) What changes in content and composition occur during metamorphosis? and (iii) What lipid classes and fatty acids provide the main metabolic energy sources during metamorphosis?

MATERIALS AND METHODS

Animals. Metamorphosing bonefish larvae were collected with a beach seine in coastal lagoons of the Gulf of California, near Guaymas, Sonora, Mexico (3), during January and February 1994. Larvae were transported live to the laboratory in Guaymas and were either placed in plastic bags and frozen (−18°C) or placed in a 60-L aquarium with running, aerated seawater at ambient temperature (18°C) and allowed to continue the metamorphosis until a desired stage was reached. Before analysis, larvae were separated into three groups based on size: early [>55 mm standard length (SL) measured from tip of snout to base of caudal fin], intermediate (35–45 mm SL), and advanced (<30 mm SL). Maximum size of early larvae is about 70 mm SL (6), and metamorphosis is complete at about 25 mm SL (3). Individual wet weight is about 0.5 g in early larvae and decreases to about 0.1 g in advanced larvae (3).

Lipid extraction. Larvae from each group were pooled to give the desired total wet mass (usually between 1–14 g), and total lipid was extracted using the method of Folch *et al.* (7). Yields were determined gravimetrically after evaporating

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Abbreviations: FAME, fatty acid methyl ester; HPTLC, high-performance thin-layer chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acids; SL, standard length; TAG, triacylglycerols; TEM, transmission electron microscopy; TLC, thin-layer chromatography.

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samples at 50°C under a stream of nitrogen and cooling to room temperature in a desiccator flushed with nitrogen. Samples were dissolved in chloroform and either used immediately or stored at −18°C under nitrogen.

Lipids were separated into neutral and polar fractions on a 21 × 3 cm column of Silica Gel 60 (70–230 mesh; Merck, Darmstadt, Germany). Samples (0.1–0.2 g) were dissolved in 2 mL chloroform and applied to the column. Neutral lipids were eluted with 150 mL chloroform. The column was then washed with 100 mL chloroform/methanol (49:1, vol/vol). After monitoring the composition of the wash by thin-layer chromatography (TLC), it was combined with the neutral fraction. Finally, polar lipids were eluted with 150 mL methanol. Yields of each fraction were determined gravimetrically after evaporating the solvent under a stream of nitrogen. Samples were dissolved in chloroform (neutral fraction) or chloroform/methanol (2:1, vol/vol) (polar fraction) at a concentration of about 30 mg × mL^{-1} and stored under nitrogen at −18°C.

Lipid analysis. One-dimensional TLC was used for preliminary qualitative analysis of the lipid class composition of total lipid extracts and to monitor column separations of neutral and polar fractions. Twenty × 20 cm plates of Silica Gel 60 G, with a thickness of about 0.25 mm, were prepared. Approximately 400 μg of sample, in a volume of 10–20 μL, was applied to plates which were developed with hexane/ethyl ether/acetic acid (90:10:1, by vol) for neutral fractions and chloroform/methanol/water (65:35:4, by vol) for polar fractions. Spots were visualized by charring after spraying with an aqueous solution of 50% sulfuric acid.

High-performance TLC (HPTLC), followed by scanning densitometry of individual spots, was used for quantification of the lipid class composition of neutral and polar fractions. A known quantity of lipid, ranging from 0.1–0.3 mg, was applied to 10 × 10 cm HPTLC plates (precoated Silica Gel 60). Neutral lipids were separated in one dimension as described previously for qualitative separations. Polar lipids were separated by two-dimensional HPTLC. The solvent for the first dimension was chloroform/methanol/28% aqueous ammonia (65:35:5, by vol); chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol) was used for the second dimension. Spots were visualized by charring after spraying with an aqueous solution of 55% sulfuric acid/0.6% sodium dichromate. Spots were identified by running known standards. Lipid classes were quantitated by scanning densitometry using VISAGE Electrophoresis Gel Analysis System software (Millipore Corp., Milford, MA) on a Sun Microsystems (Mountain View, CA) Computer linked to an Aristo Image Acquisition Console.

Phospholipids also were identified in separate, two-dimensional HPTLC runs by spraying with the phosphate group stain of Dittmer and Lester (8) as modified by Vaskovsky and Kostetsky (9); ethanolamine glycerophospholipids were visualized by spraying separate plates with 0.2% (wt/vol) ninhydrin in *tert*-butyl alcohol/water (4:1, vol/vol) (10).

Fatty acid methyl esters (FAME) were prepared from both neutral and polar fractions by acid-catalyzed transmethylation using 3 N methanolic HCl (10). Prior to the procedure, 0.22 mg of internal standard (17:0) was added to samples containing 1.5–3.0 mg total lipid in order to quantitate individual fatty acids. FAME, dissolved and stored in hexane, were separated and analyzed on a Hewlett-Packard (Avondale, PA) gas chromatograph (Model 5840 A) using an Omegawax 320 capillary column (length, 30 m; i.d., 0.32 mm; Supelco, Inc. Bellefonte, PA) of fused silica (film thickness: 0.25 μm). Nitrogen was used as the carrier gas under isothermal (190°C) conditions. Fatty acids were identified by comparison of column retention times with known standards. Peak quantification was performed with a Hewlett-Packard GC Terminal recorder and peak integrator.

Electron microscopy. Metamorphosing larvae were fixed whole by immersion in filtered seawater containing 4% paraformaldehyde and 1% glutaraldehyde (11). Fixed larvae were measured, rinsed with distilled water, and transversely sectioned with a razor blade at mid-body. Sections approximately of 2-mm thickness were rinsed with distilled water, postfixed in 1% osmium tetroxide/0.15% ruthenium red (aqueous solution), and prepared for transmission electron microscopy (TEM) as described previously (11). Thin sections (90–150 nm) were viewed and photographed on either an H-500 Hitachi (Tokyo, Japan) or a JEOL (Tokyo, Japan) 100CX transmission electron microscope.

Chemicals. Reagents used for lipid extraction and analysis were analytical grade (J.T. Baker, Phillipsburg, PA) and were purified by redistillation (12) when required. Standards used for HPTLC were from Sigma Chemical Co. (St. Louis, MO). Fatty acid standards used for gas chromatography were from Supelco, Inc. (Bellefonte, PA) and from Sigma; methanolic HCl was from Supelco.

RESULTS

Lipid extractions were performed on eight separate groups of metamorphosing bonefish leptocephali—three each on early and advanced larvae, and two on intermediate larvae. Mean total lipid content per larva (individual values in parentheses) was 7.8 (6.5, 8.3, 8.5), 5.6 (5.2, 6.1), and 4.2 (3.7, 3.8, 5.1) mg for early, intermediate, and advanced larvae, respectively, representing a net loss of about 3.6 mg, or 46%, of total lipid during metamorphosis. Mean individual wet weights for the three groups were 0.50, 0.33, and 0.20 g, respectively. Single-factor analysis of variance showed that mean values for lipid content in early and advanced larvae were significantly different at the 5% level.

In order to estimate total lipid levels as percent larval dry weight, wet weight in grams (x) of metamorphosing larvae was converted to dry weight in milligrams (y) using the regression equation: $y = 18.445 + 59.610x$; $r^2 = 0.76$; $n = 40$ (2). The resulting lipid levels were 16.2, 14.7, and 13.8% dry weight for early, intermediate, and advanced larvae, respectively.

Preliminary one-dimensional TLC analysis (not shown) of total lipid extracts of metamorphosing larvae showed the presence of triacylglycerols (TAG), cholesterol, cholesteryl esters, and phospholipids.

Neutral lipids predominated at all metamorphic stages, comprising approximately two-thirds of the total in each group (Table 1). TAG were the principal neutral lipid component in each group, but absolute amounts of TAG decreased by about 1.8 mg per larva, or about 50%, during metamorphosis, a percentage decrease similar to that seen in total lipid. Cholesterol showed little change during metamorphosis, but levels of cholesteryl esters tended to decrease slightly. Free fatty acids were not seen in the neutral fraction from any of the metamorphic stages.

Lipid class analyses of polar fractions indicated that phosphatidylethanolamine (PE) accounted for more than 50% of the total in early metamorphosing larvae, but that about 65%, or 1.0 mg, of PE was lost during metamorphosis (Table 1). Especially interesting was the apparent absence of phosphatidylcholine (PC) in early larvae. However, PC content showed a progressive increase during metamorphosis (Table 1), and in advanced larvae it accounted for about 9% of total polar lipids, although PE still predominated (35% of total). Table 1 shows also that free fatty acids were found in the polar fraction and that their content increased threefold during metamorphosis, accounting for 35% of the total in advanced larvae. Free fatty acids in the polar fraction most likely did not arise from breakdown of phospholipids on storage. Both neutral and polar fractions were stored under identical conditions before analysis, and no free fatty acids were evident in the neutral fraction. Sphingomyelin and cardiolipin were minor components and showed no apparent changes in content during metamorphosis.

Neutral lipids of early metamorphosing larvae were composed principally of polyunsaturated fatty acids (PUFA) (46.7%), followed by saturated (33.6%) and monounsaturated (18.9%) fatty acids (Table 2). These proportions showed little change during metamorphosis, although total fatty acid content decreased by 1.11 mg, or about 38%. Principal fatty acids in the neutral fraction of early-metamorphosing larvae were 16:0 (22.3%), 22:6n-3 (17.4%), 20:5n-3 (10.0%), 16:1n-7 (8.2%), and 18:1n-9 (6.4%); these percentages also showed little change during metamorphosis, although individual amounts decreased, together accounting for a loss of 752 μg, or about 68% of the loss. The fatty acid showing the largest change was 16:0, which decreased by 307 μg, or almost half, accounting for 28% of the loss. Next in importance was 22:6n-3, which decreased by 194 μg, accounting for 17% of the loss.

Polar lipids of early-metamorphosing larvae were composed principally of saturated fatty acids (50.0%), followed by PUFA (40.1%) and monounsaturated fatty acids (9.9%) (Table 3). In advanced larvae, saturated fatty acid content decreased to 40.1% and PUFA increased to 49.6%; monounsaturated fatty acids showed little change. The saturated fatty acid, 16:0, predominated in the polar fraction of early-metamorphosing larvae, accounting for 38.0% of the total, followed by 22:6n-3 (24.7%) and 18:0 (9.7%). Total fatty acid content of the polar fraction showed a 519 μg (2.6×) increase during metamorphosis, with most (33.7%) of the increase due to 22:6n-3 and 16:0 (23.5%). A significant fraction (300 μg) of the increase can be attributed to free fatty acids (Table 1).

In order to assess overall fatty acid metabolism during metamorphosis, net change for each fatty acid was calculated by subtracting its content in advanced larvae from the content in early larvae, for both neutral (Table 2) and polar (Table 3) fractions, and then summing the differences. Except for negligible increases in 18:0, 20:3n-6, and unidentified compounds (3–5 μg per larva for each), all fatty acids showed net decreases. Total loss (ignoring the aforementioned increases) was 608 μg per larva. Percentage losses for saturated, PUFA, and monounsaturated fatty acids were 44.2, 32.4, and 23.4%, respectively. The greatest loss (30.4% of the total) was seen in 16:0, followed by 14:0 (13.8%), 16:1n-7 (12.2%), 20:5n-3 (7.7%), and 18:1n-9 (7.4%). In addition to 20:5n-3, most of the net loss in PUFA was seen in 18:4n-3 (6.9%), 18:3n-3 (4.3%), and 20:3n-3

TABLE 1
Lipid Class Composition of Early, Intermediate, and Advanced Metamorphosing Bonefish Larvae Expressed as Amount per Larva (mg) and Percent Total Lipid (%)

	Stage of metamorphosis					
	Early		Intermediate		Advanced	
Lipid class	mg	%	mg	%	mg	%
Neutral fraction						
Triacylglycerols	3.49	44.7	2.19	39.2	1.72	40.9
Cholesteryl esters	0.83	10.6	0.71	12.7	0.58	13.8
Cholesterol	0.30	3.9	0.34	6.0	0.25	5.9
Unidentified	0.39	5.0	0.28	5.0	0.15	3.5
Total neutral	5.0	64.2	3.5	62.9	2.7	64.1
Polar fraction						
Free fatty acids	0.15	1.9	0.31	5.5	0.45	10.7
Phosphatidylcholine	n.d.[a]	0.0	0.05	0.9	0.13	3.1
Phosphatidylethanolamine	1.53	19.6	0.99	17.6	0.53	12.6
Sphingomyelin	0.26	3.3	0.27	4.9	0.25	5.9
Cardiolipin	0.14	1.8	0.19	3.4	0.05	1.1
Unidentified	0.72	9.2	0.27	4.8	0.11	2.5
Total polar	2.8	35.8	2.1	37.1	1.5	35.9

[a]Not detected.

TABLE 2
Change in Fatty Acid Content (μg × larva^{-1}) of Neutral Lipid Fraction During Metamorphosis of Bonefish Larvae[a]

	Stage of metamorphosis					
	Early		Intermediate		Advanced	
Fatty acid	μg	%	μg	%	μg	%
14:0	211 ± 14	7.1	157 ± 8	6.9	119 ± 7	6.4
14:1	38 ± 4	1.3	33 ± 1	1.4	24 ± 1	1.3
16:0	661 ± 22	22.3	459 ± 16	20.0	354 ± 9	19.1
16:1n-7	244 ± 29	8.2	197 ± 12	8.6	157 ± 3	8.5
18:0	125 ± 4	4.2	108 ± 2	4.7	85 ± 1	4.6
18:1n-9	190 ± 20	6.4	155 ± 3	6.8	122 ± 2	6.6
18:1n-7	90 ± 7	3.0	78 ± 3	3.4	62 ± 0	3.3
18:2n-6	80 ± 11	2.7	65 ± 2	2.8	54 ± 1	2.9
18:3n-3	125 ± 11	4.2	106 ± 3	4.6	92 ± 2	5.0
18:4n-3	143 ± 14	4.8	126 ± 2	5.5	99 ± 2	5.3
20:3n-6	27 ± 2	0.9	22 ± 0	1.0	17 ± 1	0.9
20:3n-3	30 ± 12	1.0	12 ± 0	0.5	9 ± 1	0.5
20:4	89 ± 6	3.0	77 ± 2	3.4	67 ± 3	3.6
20:5n-3	297 ± 17	10.0	243 ± 3	10.6	201 ± 6	10.8
22:4n-6	28 ± 1	1.0	19 ± 1	0.8	14 ± 3	0.8
22:5n-3	51 ± 0	1.7	35 ± 1	1.5	30 ± 6	1.6
22:6n-3	516 ± 0	17.4	368 ± 0	16.0	322 ± 59	17.4
Unidentified	24 ± 5	0.8	34 ± 2	1.5	27 ± 3	1.4
Total	2,969	100	2,294	100	1,855	100
Saturated	997	33.6	724	31.6	558	30.1
Monoenes	562	18.9	463	20.2	365	19.7
PUFA	1,386	46.7	1,073	46.8	905	48.8
n-3/n-6	8.61		8.40		8.86	

[a]Values for individual fatty acids are means ± SE of three determinations, except for the three fatty acids of the C_{22} series, where values are means and ranges of two determinations. Mean values for individual fatty acids of early and advanced larvae, with the exception of those from the C_{22} series, were analyzed by single-factor analysis of variance; all values, except those for 18:2n-6 and 20:3n-3, were significantly different at the 5% level, PUFA, polyunsaturated fatty acids.

TABLE 3
Change in Fatty Acid Content (μg × larva^{-1}) of Polar Lipid Fraction During Metamorphosis of Bonefish Larvae[a]

	Stage of metamorphosis					
	Early		Intermediate		Advanced	
Fatty acid	μg	%	μg	%	μg	%
14:0	8 ± 1	2.4	9 ± 1	1.3	16 ± 2	1.9
14:1	n.d.	0.0	n.d.	0.0	n.d.	0.0
16:0	126 ± 18	38.0	188 ± 8	26.7	248 ± 18	29.2
16:1n-7	10 ± 3	3.0	13 ± 4	1.9	23 ± 3	2.7
18:0	32 ± 1	9.7	77 ± 2	10.9	77 ± 1	9.1
18:1n-9	12 ± 0	3.6	22 ± 1	3.1	35 ± 1	4.1
18:1n-7	11 ± 0	3.3	26 ± 1	3.7	30 ± 0	3.5
18:2n-6	4 ± 0	1.2	9 ± 1	1.3	12 ± 0	1.4
18:3n-3	4 ± 0	1.2	7 ± 0	1.0	11 ± 0	1.3
18:4n-3	n.d.	0.0	n.d.	0.0	2 ± 2	0.2
20:3n-6	9 ± 0	2.7	20 ± 1	2.9	24 ± 1	2.8
20:3n-3	2 ± 2	0.6	15 ± 0	2.1	1 ± 1	0.1
20:4	7 ± 1	2.1	19 ± 1	2.7	22 ± 1	2.6
20:5n-3	20 ± 1	6.0	41 ± 2	5.8	69 ± 3	8.1
22:4n-6	n.d.	0.0	8 ± 0	1.1	8 ± 1	0.9
22:5n-3	5 ± 0	1.5	15 ± 1	2.1	16 ± 1	1.9
22:6n-3	82 ± 0	24.7	235 ± 8	33.4	257 ± 23	30.2
Total	332	100	704	100	851	100
Saturated	166	50.0	274	38.9	341	40.1
Monoenes	33	9.9	61	8.7	88	10.3
PUFA	133	40.1	369	52.4	422	49.6
n-3/n-6	8.69		8.46		8.09	

[a]Values for individual fatty acids are means ± SE of three determinations, except for the three fatty acids of the C_{22} series, where values are means and ranges of two determinations. Mean values for individual fatty acids of early and advanced larvae, with the exception of those from the C_{22} series, were analyzed by single-factor analysis of variance; all values, except those for 14:1, 18:4n-3, and 20:3n-3, were significantly different at the 5% level. Abbreviations as in Tables 1 and 2.

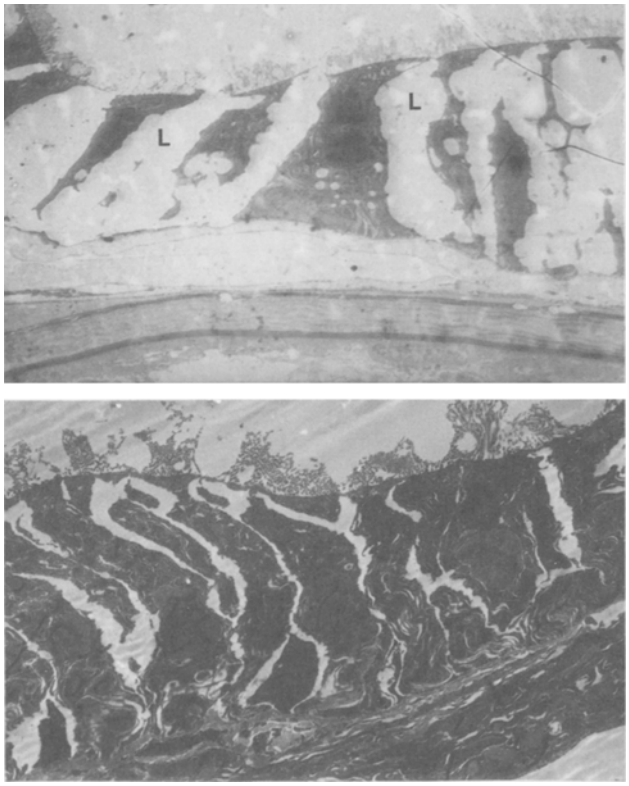


FIG. 1. Transmission electron micrograph of midgut epithelium of early [61 mm standard length (SL); upper] and advanced (26 mm SL; lower) metamorphosing leptocephali of the bonefish *Albula* sp. Note the abundance of intercellular lipid deposits (L) in early larvae and the decrease in these deposits in advanced larvae. Midgut lumen is at the top of each figure. Magnification: × 1,504 (upper); × 6,284 (lower).

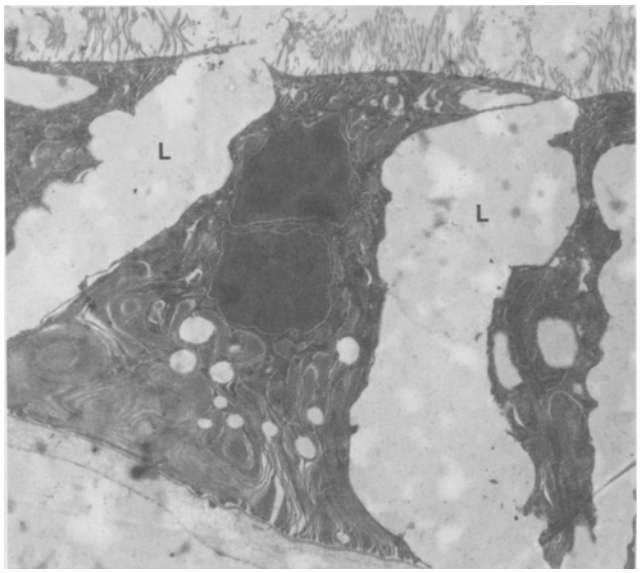


FIG. 2. Higher-power transmission electron micrograph of an early (61 mm SL) metamorphosing leptocephalus of the bonefish *Albula* sp. showing details of the ultrastructure of the midgut epithelial cell seen in the center of Figure 1 (upper). Note the extensive system of invaginations of the basal membrane which contains lipid droplets, the numerous mitochondria closely associated with these droplets, intercellular lipid deposits (L), and well-developed microvilli of the apical membrane. Magnification: × 3,995.

(3.6%). The principal PUFA, 22:6n-3, in both neutral and polar fractions of early-metamorphosing larvae (Tables 2 and 3), showed a net loss of only 19 μg per larva (3.1%).

TEM (Figs. 1 and 2) revealed that part of the lipid catabolized during metamorphosis arose from deposits located in midgut epithelium. Early-metamorphosing larvae (Fig. 1, upper) showed abundant osmiophobic areas among the epithelial cells, typical of lipid with high saturated fatty acid content. Relative amounts of lipid showed a progressive decrease in intermediate (not shown) and advanced (Fig. 1, lower) metamorphosing larvae. Higher magnification of a midgut epithelial cell of early metamorphosing larvae (Fig. 2) also showed the presence of apparent lipid droplets in channels formed by extensive infoldings of the basal membrane. These droplets, not found in all cells, were localized predominantly in the basal area and were closely associated with abundant mitochondria, some of which were very large, slender, and curved. The abundance of mitochondria suggests that these cells are very active metabolically.

DISCUSSION

The present study has provided a clearer picture of lipid metabolism in metamorphosing leptocephali of the bonefish. The total amount of lipid catabolized per larva agrees well with results from an earlier study (2). Although neutral lipid is deposited in the extracellular gelatinous body matrix during early (premetamorphic) larval development (5), and is the most likely source of the majority of lipid mobilized during metamorphosis, we have shown that highly-saturated lipid deposits in the midgut region also are utilized.

Ultrastructure of midgut epithelial cells of early-metamorphosing bonefish leptocephali was similar to that described for premetamorphic eel (*Conger myriaster*) leptocephali (13). However, no evidence was seen for either lipid droplets within basal infoldings or for intercellular lipid deposits in *C. myriaster*, suggesting that in bonefish these may be deposited just prior to metamorphosis. This possibility is supported by results on the eel *Ariosoma balearicum*, where it was found that most of the whole-body lipid is deposited during the latter part of the premetamorphic interval (14). In addition, the level of total lipid (4.0 mg × larva^{-1}) in a single extract from relatively advanced premetamorphic bonefish leptocephali was about half that seen in early-metamorphosing larvae (Padrón, D., and Pfeiler, E., unpublished data), again suggesting that a significant fraction of lipid deposition occurs relatively late in the premetamorphic interval.

Whole-body lipid content in early-metamorphosing bonefish larvae, approximately 16% of total dry weight (2; this study), is similar to that of tarpon (*Megalops atlanticus*) and ladyfish (*Elops saurus*) leptocephali (Donnelly, J., Torres, J.J., and Crabtree, R.E., unpublished data), as well as to that of yolk-sac larvae of other teleost fishes (15–17). In addition to

providing the principal source of energy during bonefish metamorphosis, lipid also probably functions as a hydrostatic agent as premetamorphic and early metamorphic larvae lack a functional swimbladder (4). However, lipid content alone is insufficient to achieve neutral buoyancy because leptocephali sink when swimming movements cease (4). The swimbladder does not become functional until metamorphosis, and lipid breakdown, is already underway (4). Although about 50% of total lipid content is lost during metamorphosis, lipid levels expressed as percentage dry weight show little change (2; this study) because about 50% of total larval dry weight also is lost (3).

The principal lipid class broken down during metamorphosis was TAG. All classes of fatty acids (saturated, monounsaturated, and PUFA) were utilized and probably contributed to energy production. Although major decreases in PUFA occurred during metamorphosis, and relatively large amounts of 22:6n-3 were lost from the neutral fraction as TAG were degraded, most (90%) of this fatty acid was conserved.

In both neutral and polar fractions, n-3/n-6 ratios were high (8.1–8.9) and relatively constant during metamorphosis. High n-3/n-6 ratios (>8.0) are typical of neutral and polar lipids of eggs and larvae of marine fishes (18–20).

Although PC was not evident in early-metamorphosing larvae, it could have been present at levels below our detection limits. Another possibility is that PC was present, but lost during column separations; the observation that PC was present in intermediate and advanced larvae, showing a progressive increase in level during metamorphosis (Table 1), argues against this interpretation.

The source of PE that was catabolized during metamorphosis is unknown, but histochemical tests indicate that phospholipids are absent from the gelatinous matrix (5). Some PE may be released from membranes of degraded skin epithelial cells. Skin surface area decreases by approximately 75% during metamorphosis (see Fig. 1 in Ref. 2), and scanning electron microscopy and TEM of skin of metamorphosing larvae have demonstrated the presence of degenerating cells (11), reminiscent of cells undergoing "programmed cell death" or apoptosis (21).

Although many studies have dealt with changes in lipid class and fatty acid composition during embryonic and larval development in teleost fishes (e.g., Refs. 15–17,22), no previous data are available for developing leptocephali, a unique life history stage found only in fishes of the subdivision Elopomorpha, which includes bonefishes, tarpon, and true eels (4). However, lipid class and fatty acid composition of postmetamorphic "glass eels" of the American eel (*Anguilla rostrata*) are similar to those of advanced metamorphic bonefish larvae (23). This suggests that patterns of lipid class and fatty acid metabolism during metamorphosis of eels also may be similar, although this remains to be shown.

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Purification and Characterization of Deep Sea Shark *Centrophorus squamosus* Liver Oil 1-*O*-Alkylglycerol Ether Lipids

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ABSTRACT: The 1-*O*-alkylglycerol composition of the liver oil of the deep sea shark *Centrophorus squamosus*, a species which provides edible flesh, has been determined. After various fractionations of the oil, the unsaponifiable fraction was characterized by means of gas chromatography/mass spectrometry, electron impact, and positive-ion chemical ionization. The oil is composed of 60% unsaponifiable matter, containing 45% squalene, 4.5% cholesterol, and 10% of linear saturated and monounsaturated glycerol ethers with 14–18 carbon atoms. After a first separation by chromatography on silicic acid, monounsaturated glycerol ethers have been separated from the saturated homologues, in particular from 1-*O*-octadecylglycerol (batyl alcohol) and 1-*O*-hexadecylglycerol (chimyl alcohol), *via* urea complexation. This newer application of the urea method, already used in the past to extract saturated from polyunsaturated fatty acids, allowed the purification of the main components of the complex unsaturated glycerol ether fraction, namely, 1-*O*-octadecen-9'ylglycerol (selachyl alcohol) and 1-*O*-hexadecen-9'ylglycerol.
Lipids 31, 521–528 (1996).

The deep sea shark *Centrophorus squamosus* (1) lives at depths below 1000 m; it can provide large quantities of squalene-rich liver oil, because this fish, which can reach 1 m in length, has a liver comprising 50% of body weight (2). We recently reported (3) the characterization of fatty acid methyl esters from this liver oil by gas chromatography/mass spectrometry (GC/MS), and the purification of two esters each of docosahexaenoic acid (DHA or 22:6) and of docosamonoenoic acid (DMA or 22:1), by countercurrent chromatography. We then directed our investigations to the complementary unsaponifiable fraction, more particularly to the 1-*O*-alkylglycerol fraction, because of its potential applications in industry.

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Abbreviations: APRL, antihypertensive polar renomedullary lipid; DHA, docosahexaenoic acid; DMA, docosamonoenoic acid; EI, electron impact; EPA, eicosapentaenoic acid; FID, flame-ionization detector; GC/MS, gas chromatography/mass spectrometry; GE, glycerol ethers; PAF, platelet-activating factor; PCI, positive-ion chemical ionization; TLC, thin-layer chromatography; TMS, trimethylsilyl radical. The abbreviation "en-n'yl" for 1-*O*-alkylglycerols indicate a normal *cis* ethylenic bond on the *nth* carbon from the ether oxygen.

The unsaponifiable matter of this liver oil contains squalene and glycerol ethers. Squalene is used as a food additive and as a precursor of squalane; the latter compound is utilized largely as an excipient in cosmetics and pharmaceuticals (4). Glycerol ethers, including those of chimyl or batyl alcohol (or their monounsaturated derivatives), have enormous economic potential because they exhibit bacteriostatic and fungistatic properties (5,6), anti-inflammatory activities (7), and hemopoietic effects (8,9). They are also believed to protect against radiation damage and to possess antitumor properties (10). With the recent interest in platelet-activating factor (PAF) (11,12), and the antihypertensive polar renomedullary lipid (APRL) (13), both of which contain a glycerol ether in their structure, ether lipids can also be considered as biochemical mediators (14,15).

MATERIALS AND METHODS

Shark oil. Centrophorus squamosus liver oil was obtained from the IS-FRANCE Company (Lorient, France).

Standards. Racemic batyl alcohol came from Fluka (Buchs, Switzerland) and (+) batyl alcohol from Extrasynthese (Genay, France). Squalene originated from Robeco Chemicals, Inc. (New York, NY) and cholesterol from Janssen Chimica (Geel, Belgium).

Chemicals. AR Normapur solvents and salts used came from Prolabo (Paris, France). The BF_3 was purchased from Fluka, chlorotrimethylsilane from Janssen Chimica, and *N,O*-Bis(trimethylsilyl)acetamide from Aldrich Chemie (Steinheim, Germany).

Shark liver oil fractionation. The saponification was performed according to the procedure recommended by the *Pharmacopée Française* (16). Crude oil (20 g) was diluted with 20 mL of heptane and 40 mL of methanol with 4.5 g of KOH (dissolved with heating); the reaction was carried out under magnetic stirring at 70°C for 30 min.

The potassium salts of fatty acids produced after the saponification were converted into the corresponding methyl esters by adding 150 mL of BF_3 in methanol (10%, by wt) (17); the reaction was achieved with magnetic stirring at 70°C for 15 min. After addition of 170 mL of heptane, the solution was washed with an equal volume of water saturated with

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NaCl and dried over Na_2SO_4. An oily mixture (20 g), of a pale yellow color, including both neutral and polar nonfatty acid materials, was obtained after evaporation of the solvent.

Separation of the neutral and polar materials. Esters and other materials (10 g) were submitted to chromatography on a 30 cm × 3 cm column filled with 100 g of silicic acid (ASTM, 230–400 mesh; Merck, Darmstadt, Germany). The eluent was a mixture of heptane and diethyl ether of increasing polarity (18). Five fractions (F1, F2, F3, F4, and F5) were obtained; the final fraction (F5), corresponding to very polar compound(s), was eluted with methanol; the methanol of the last fraction was evaporated and diethyl ether added to the residue, the solution was then filtered on a Büchner funnel to eliminate silica residues.

Collected fractions were analyzed by thin-layer chromatography (TLC) on silica gel 60 F_{254} Special from Riedel-de-Haën (Seelze, Germany), with two different eluents. The less polar fractions were eluted with a mixture of heptane/diethyl ether/formic acid (1:0.25:0.025, by vol), the more polar with the same mixture in the proportions (0.25:1:0.025, by vol) (19). Compounds were visualized by spraying with H_2SO_4 and heating; results are shown in Tables 1 and 2.

Silylation of glycerol ether and cholesterol fractions. Nonvolatile compounds such as 1-*O*-alkylglycerols (F4) and cholesterol (F3) had to be converted into more volatile silyl ethers prior to GC, which is a technique well suited for the analysis of molecules of this kind (20). Silylation was performed by adding 200 µL of *N,O*-Bis(trimethylsilyl)acetamide and 100 µL of chlorotrimethylsilane to 10 mg of such compound(s); the reaction was carried out at 70°C for 20 min with magnetic stirring. After addition of 200 µL of diethyl ether 1 µL of each silylated fraction was injected into the GC.

Analysis of F1, F3, and F4 by GC. Unsaponifiable fractions were first examined by GC to optimize the conditions for further analysis by GC/MS. The analytical system was a Shimadzu Model GC-14A, connected to a flame-ionization detector (FID), controlled with a chromatopac C-R5A integrator (Shimadzu Corporation, Kyoto, Japan), and equipped with a nonpolar dimethylpolysiloxane CP-Sil 5 CB (Chrompack, Delft, The Netherlands) fused-silica capillary column (25 m × 0.25 mm × 0.12 µm). The final optimized analytical conditions were: T_{inj} and T_{det} 285°C, T_{col} isothermal at 190°C for 5 min followed by a programmed temperature gradient from 190 to 285°C at 10°C/min^{-1}, P_{He} 10^5 Pa, split of 80 mL/min, injected quantity 1 µL of silylated compound(s) dissolved in diethyl ether or (20 g/L) for the fraction(s) which were not silylated. See Figure 1 for the results for F4.

Analysis of F1, F3, and F4 by GC/MS. The structures of compounds were characterized by GC/MS experiments with a combination of a chromatograph gas Varian Model 3000 (Sunnyvale, CA), using the same column and conditions as previously described, and a Nermag R10-10C mass spectrometer controlled by a Digital PDP11-23 Plus System (Delsi-Nermag, Argenteuil, France). Electron impact (EI) spectra were recorded at 70 eV, and positive-ion chemical ionization (PCI) studies were performed with NH_3 (Air Liquide, Le Plessis-Robinson, France) as reagent gas at a pressure of 0.1 Torr (1 Torr = 133.3 Pa) in the ion source.

Purification of monounsaturated glycerol ethers (GE) of F4 by urea complexation of saturated GE. Five grams of GE fraction (F4) was dissolved at 70°C in 50 mL of ethanol and 10 mL of methanol, with 5, 15, or 30 g of urea, corresponding, respectively, to the weight ratios $R = 1$, $R = 3$, and $R = 6$ (with R = U/F4, by wt). Urea and urea complexes were allowed to crystallize overnight at 4°C. The crystallized material was separated by filtration on a Büchner funnel.

To recover the noncomplexing materials held in solution, 60 mL of water was added to each filtrate. Then, 6 N HCl was added, with magnetic stirring, until the mixture reached a pH of 4–5. Each solution was then extracted with 2 × 100 mL of diethyl ether. The extracts were separately dried over Na_2SO_4 and filtered. After evaporation of the solvent, a white wax was obtained for each extract of the three filtrates.

Each urea complex precipitate was also dissolved in 150 mL of water and neutralized by 6 N HCl to pH 4–5. Solutions were individually extracted with 2 × 100 mL of diethyl ether and dried over Na_2SO_4. After filtration and solvent evapora-

TABLE 1
Thin-Layer Chromatography of the Crude, Saponified, and Esterified Deep Sea Shark Liver Oil with the Less Polar Eluent: *n*-Heptane/Diethyl Ether/Formic Acid (1:0.25:0.025, by vol)

R_f	Crude oil	Saponified oil	Esterified oil
0.95	Squalene	Squalene	Squalene
0.90	Cholesterol esters		
0.65			Fatty acid methyl esters
0.57	Diacyl-, monoalkylglycerols		
0.41	Triacylglycerols		
0.27	Fatty acids	Fatty acids	
0.14	Cholesterol	Cholesterol	Cholesterol
0.06	monoacyl-, monoalkylglycerols		
0.01	Diacylglycerols		
0.00	X[a] (Polar compounds)	Monoalkylglycerols, X	Monoalkylglycerols, X

[a]X for undetermined compounds.

TABLE 2
Composition and Percentages of the Different Fractions Obtained After Chromatography on Silica Gel of the Esterified Deep Sea Shark Liver Oil

Fraction	Compound	Percentage (%)
F1	Squalene	45.0
F2	Fatty acid methyl esters	40.0
F3	Cholesterol	4.5
F4	1-*O*-alkylglycerols	10.0
F5	X[a]	0.5
Esterified deep sea shark liver oil		100

[a]X for polar compounds eluted with methanol.

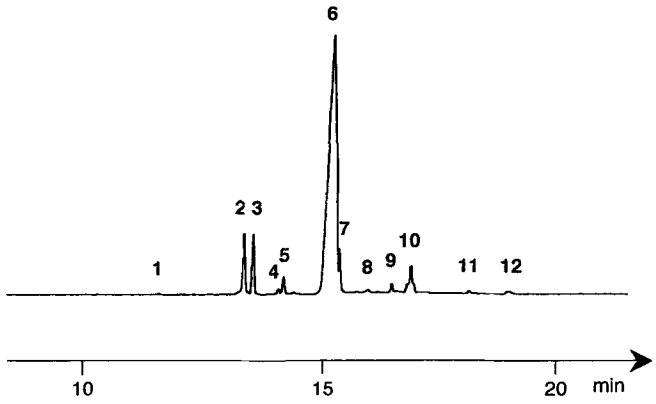


FIG. 1. Gas chromatogram of glycerol ethers of the silylated fraction F4. Column: CP-Sil 5 CB (25 m × 0.25 mm × 0.12 μm); pressure: 10^5 Pa; column temperature: 190°C for 5 min then 190 to 285°C at 10°C/min.

tion, pale yellow waxes were obtained. All diethyl ether extracts were silylated prior to GC, following the experimental procedure previously described.

RESULTS

Chromatography of C. squamosus *liver oil products on silica gel.* The original crude, deep sea shark liver oil, the saponified oil, and the esterified materials were first analyzed by TLC. Spots were compared with standards and with TLC profiles of other oils found in the literature (21). Table 1 shows that crude oil is composed of three kinds of molecules, i.e., squalene, free cholesterol, and fatty acids as various esters of glycerol or 1-*O*-alkylglycerols. Saponification, followed by esterification, leads to a mixture of squalene, fatty acid methyl esters, cholesterol, and the monoalkylglycerols. The monoalkylglycerols were easily recovered by column chromatography, as shown in Table 2. It appears that this species of deep sea shark has a liver oil which contains an important amount of unsaponifiable matter (60%), of which a very large proportion is squalene (see Table 2). This marine oil also contains significant amounts of ether lipids (10%) and a small quantity of cholesterol (4.5%).

F1, F3, and F4 GC/MS analysis. To confirm the composition of the unsaponifiable matter of the deep sea shark liver oil, fractions F1, F3, and F4 (as defined in Table 2), were first analyzed by GC, then by GC/MS experiments. GC analysis of fraction F1 showed only one chromatographic peak with a retention time of 17.69 min identical with the retention time of a squalene standard. This was confirmed by comparing the EI spectrum of F1 with that of squalene, given by the library (22). Both spectra showed the following characteristic fragments: $[M]^{+\bullet}$ of *m/z* 410, $[M - CH_3]^+$ of *m/z* 395, $[M - C_3H_7]^+$ of *m/z* 367 and $[M - C_5H_9]^+$ of *m/z* 341. The molecular ion and isoprenic fragments of the spectrum were clear enough to identify the compound from F1 as squalene.

In GC experiments, silylated fraction F3 appeared as a pure compound with a single chromatographic peak with a retention time of 20.88 min, coinciding with that of the silylated cholesterol standard. The EI-spectrum of F3 was also comparable to that of silylated cholesterol given by the literature (23), confirming the previous data: *m/z* 458 represents the molecular ion of silylated cholesterol, whereas the following fragments, $[M - CH_3]^+$ of *m/z* 443, $[M - TMSOH]^{+\bullet}$ of *m/z* 368, $[M - (CH_3 + TMSOH)]^+$ of *m/z* 353, $[M - (C_3H_4 + TMSO)]^+$ of *m/z* 329, and $[M - (TMSOH + C_9H_{13})]^+$ of *m/z* 247, (TMS for trimethylsilyl radical) were also present.

GC/MS analysis of silylated fraction F4 demonstrated that it contained several silylated glycerol ethers. Comparison with silylated batyl alcohol standard, and with results found in the literature for 2-*O*-alkyl-1,3-di-*O*-TMS-glycerols, and di- and tri-*O*-alkyl-TMS-glycerols (24), proved that the silylated fraction F4 was mainly a mixture of GE in the 1-*O*-alkyl-2,3-di-*O*-TMS-glycerol form. The gas chromatogram and GE composition of the silylated fraction F4 are shown in Figure 1 and Table 3, respectively. The EI-spectrum of silylated batyl alcohol (Fig. 2) and those of peaks numbers 1–7 of the silylated fraction F4 gas chromatogram present the following similarities: a base peak of *m/z* 205; peak relative to the TMS radical appears in all cases; ions derived from the glycerol-2,3-di-*O*-TMS moiety, namely, *m/z* 147, 133, 130, 117, and 103 are present with 5–25% relative intensity; (M –

TABLE 3
Composition of Glycerol Ethers of the Silylated Fraction F4 Determined from the Gas Chromatogram (GC) of Figure 1

GC peaks	Retention time (min)	%	Compounds[a]
0	2.59	—	Solvents
1	11.54	1.0	14:0
2	13.29	6.0	16:1
3	13.47	5.0	16:0
4	13.97	0.5	17:1
5	14.08	1.5	17:0
6	15.11	76.0	18:1
7	15.12	3.0	18:0
8	15.76	1.0	
9	16.25	1.0	
10	16.64	4.0	X
11	17.83	0.5	
12	18.59	0.5	
Silylated fraction F4		100	

[a]Nomenclature: 16:0 for 1-*O*-hexadecylglycerol and 16:1 for 1-*O*-hexadecenylglycerol, X for undetermined compounds.

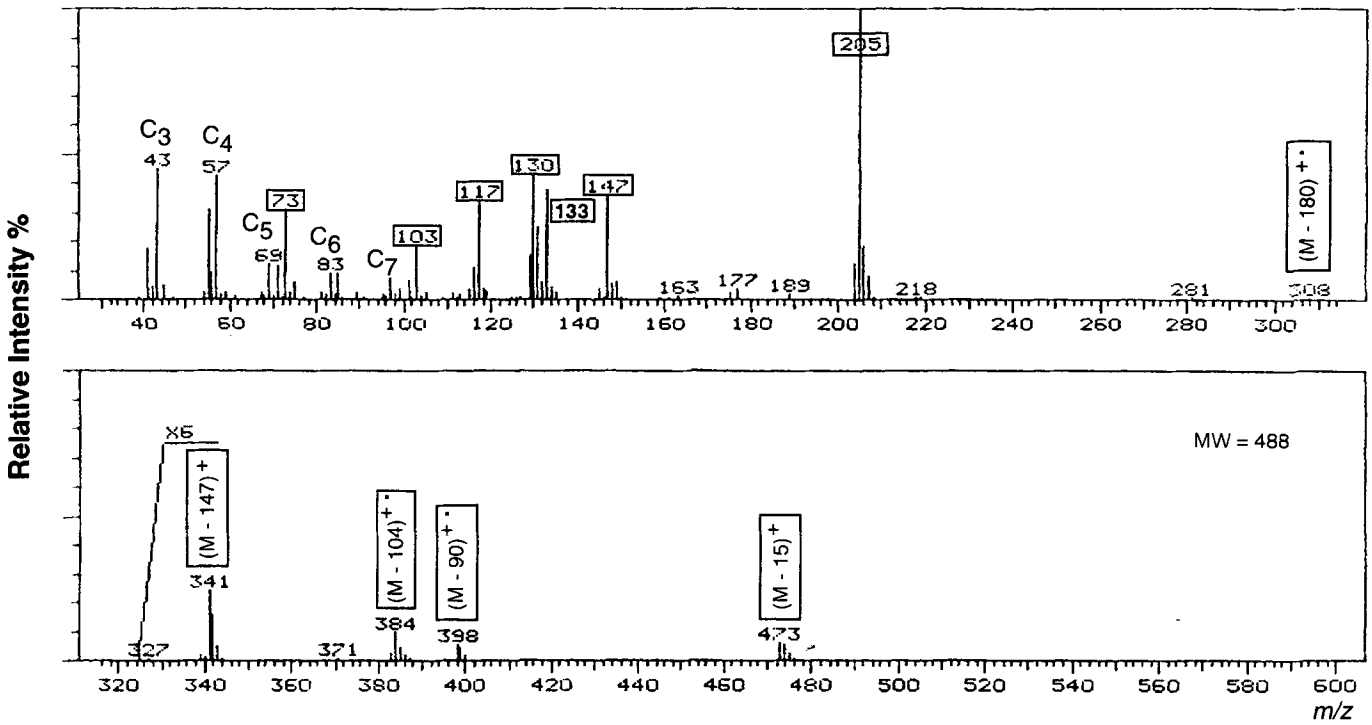


FIG. 2. Electron impact-spectrum of 1-O-octadecyl-2,3-di-O-trimethylsilyl-glycerol (silylated batyl alcohol) with its characteristic fragmentations. MW, molecular weight.

15)$^+$ is always present with 5–10% relative abundance, as well as (M – 90)$^{+\bullet}$, (M – 104)$^{+\bullet}$, (M – 147)$^+$, and (M – 180)$^{+\bullet}$ with lower relative intensities; in the lower mass range, the alkyl ions with *m/z* 43, 57, 71, 85, etc., for the saturated compounds and *m/z* 41, 55, 69, 83, etc., for the unsaturated ones, are of significant intensity; the molecular ion with low relative abundance could be observed only in unsaturated GE; the molecular weight of saturated GE was verified by PCI experiments. Assigments of fragment ions are indicated in Figure 3. Examples of EI and PCI-spectra of 1-*O*-hexadecyl-,2,3-di-*O*-TMS-glycerol and EI-spectrum of 1-*O*-hexadecenyl-,2,3-di-*O*-TMS-glycerol (Fig. 4) found in the silylated fraction F4 show the characteristic fragmentations of 1-*O*-alkyl-2,3-di-*O*-TMS-glycerols. Our GC/MS experiments demonstrate that the ether lipids of the esterified deep sea shark liver oil are a mixture of saturated and monounsaturated 1-*O*-alkylglycerols with linear alkyl chains composed of 14–18 carbon atoms, the major compound of which is 1-*O*-octadecenylglycerol. Although substituted GE, namely, 1-*O*-(2-methoxyalkyl) and 1-*O*-(2-hydroxyalkyl) (27) glycerols, were previously isolated by Hallgren *et al.* (25–27) from Greenland shark and other marine animals, we have not detected these compounds in *C. squamosus* liver oil.

*Purification of 1-*O*-octadecen-9′yl and 1-*O*-hexadecen-9′ylglycerol mixture* via *urea complexation.* Urea complexation of saturated fatty acids has been widely used for their removal from a mixture containing polyunsaturated fatty acids such as eicosapentaenoic acid (EPA or 20:5n-3) and DHA or 22:6n-3 (28,29). Due to their flexible chain, saturated molecules form urea complexes (host-guest molecules) more easily than their unsaturated homologues which have *cis* double bonds and are therefore partly rigid (30). We successfully applied this procedure to the purification of unsaturated glycerol ethers by selectively removing their saturated congeners with urea from fraction F4.

The composition of the filtrate, determined by GC, and the yield in monounsaturated GE, are shown in Figure 5 for three different ratios of urea to that of fraction F4, R = U/F4 (by wt). For each ratio R, the presence of saturated GE and other impurities in the filtrate is negligible compared with that of monounsaturated GE. Increasing the amount of urea results in a decrease in both yield and purity of the monounsaturated GE, and as a consequence does not favor their purification. This is, of course, a very important parameter in large-scale preparation, because it minimizes the amount of urea to be used. The proportion of the two main compounds found in the filtrate, namely 1-*O*-octadecen-9′yl and 1-*O*-hexadecen-9′yl-glycerol, varies with the ratio R = U/F4 as well, as shown in Figure 6. These results prove that the ratio R also has an effect on the efficiency of the purification procedure because monounsaturated GE do not have identical behaviors toward urea complexation. Behavior seems to depend upon the carbon chain length of the monounsaturated GE; a higher R does not result in discrimination based on the carbon chain length, whereas a lower R allows purification of longer monounsaturated GE from shorter ones.

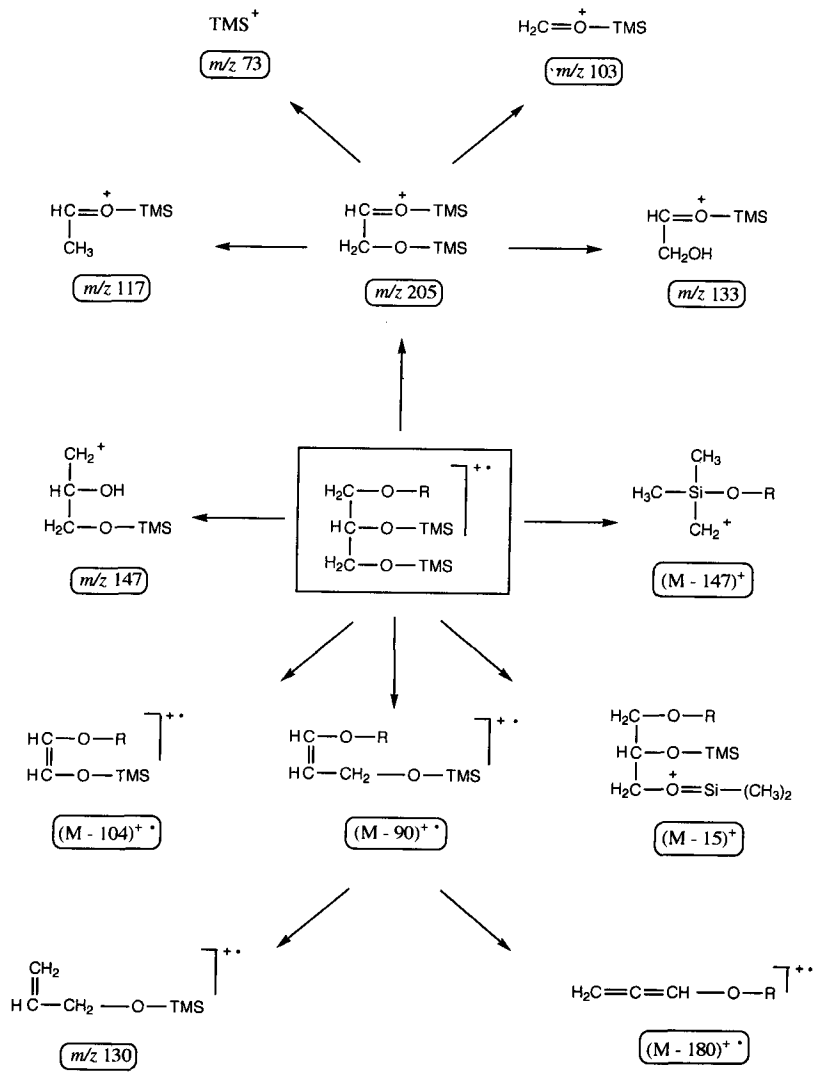


FIG. 3. Scheme of specific fragmentations of 1-*O*-alkyl-2,3-di-*O*-TMS-glycerols (TMS-trimethylsilyl radical).

DISCUSSION

Significance of the notable ether lipid and squalene contents of the liver oil of C. squamosus. Elasmobranch fish (sharks, skates and rays) usually have large livers, containing important quantities of low-density oil, presumably to serve as a substitute for the commonly found gas-filled swim bladder of teleost species (31). The liver oil of *C. squamosus* differs from that at pelagic or sedentary deep sea sharks and contains a larger proportion of low-density lipids (squalene and ether lipids) (32), probably because it is a fast-swimming deep sea shark species (33). Various hypotheses have been put forward to explain the high percentages of these compounds in the hepatic oil of this kind of fish (34). One is the possibility that the fast-moving deep sea sharks use these molecules, and particularly squalene (35), or diacyl- and monoalkylglycerols (36), to maintain an appropriate buoyancy during vertical migrations. Another explanation must be sought for the high percentage of squalene, a molecule that is highly susceptible to oxidation (37).

The use of silylated glycerol ethers to resolve the structures of these compounds of natural complex oils by GC/MS. While the potential of GC/MS for the identification of ether lipids in natural samples is enormous, there have been few reports of the use of silylated glycerol ether derivatives, although they were described by Myher *et al.* (38). Isopropylidene derivatives are usually preferred to identify 1-O-monoalkylglycerols (39). Silylated ether lipids are generally examined by capillary GC on polar liquid phases. This work demonstrates that capillary GC on a nonpolar liquid phase, coupled with MS detection of silylated ether lipids, is also an effective method which can be used to sucessfully separate and determine ether lipid compositions from complex mixtures such as natural extracts of marine origin. For many natural samples, the retention times relative to authentic standards should serve as identification guides. However, this procedure is not suitable to identify the double bond positions of such compounds, even through further use of isopropylidene derivatives on nonpolar GC phases. The unsaturation posi-

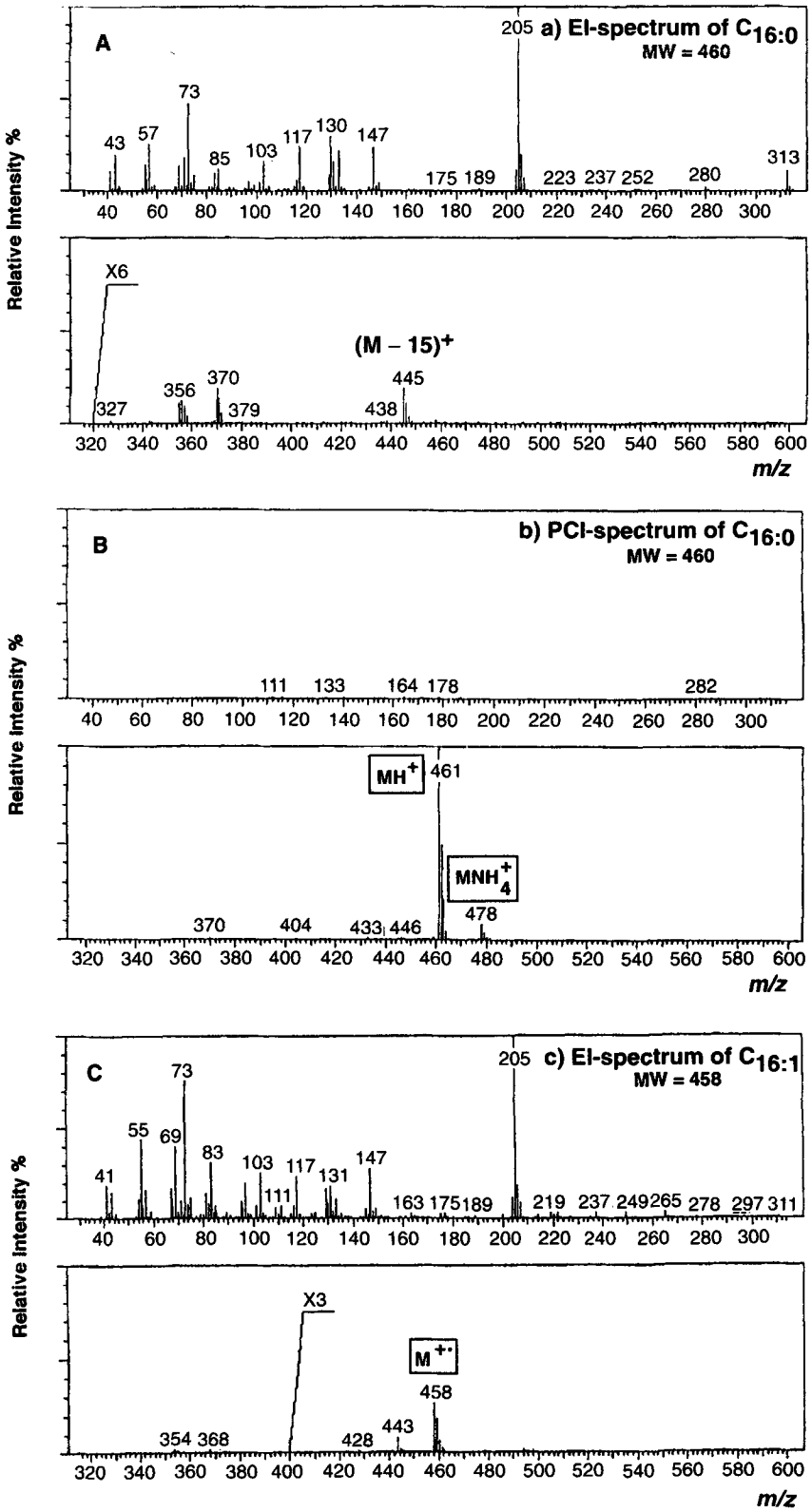


FIG. 4. A, Electron impact (EI)-spectrum of 1-*O*-hexadecyl-2,3-di-*O*-TMS-glycerol of the silylated fraction F4; E = 70 eV. B, Positive-ion chemical ionization (PCI)-spectrum of 1-*O*-hexadecyl-2,3-di-*O*-TMS-glycerol of the silylated fraction F4; reagent gas, NH_3 at 13.33 Pa. C, EI-spectrum of 1-*O*-hexadecenyl-2,3-di-*O*-TMS-glycerol of the silylated fraction F4; E = 70 eV. Abbreviations as in Figures 2 and 3.

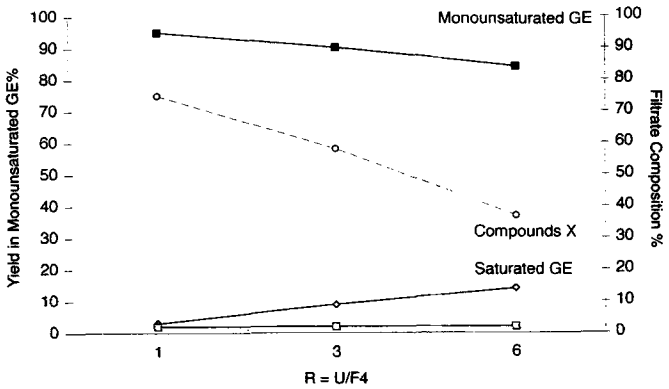


FIG. 5. Yield of monounsaturated glycerol ethers (GE) and composition of monounsaturated, saturated GE, and undetermined compounds X, obtained in the filtrate from the GE fraction F4 after overnight urea complexation at 4°C, with various amounts of urea (U), given by the ratio *R* = U/F4 (by wt); percentages acquired by gas chromatography.

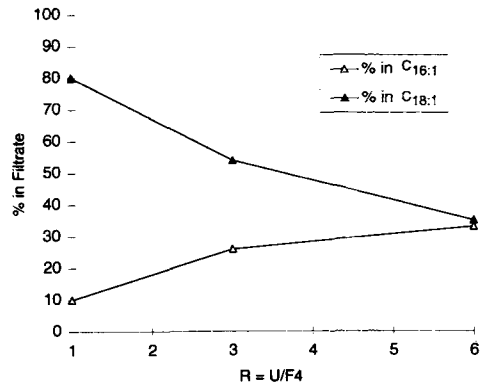


FIG. 6. Influence of the quantity of urea (U) given by the ratio *R* = U/F4 (by wt), used for the urea complexation overnight at 4°C, on the proportion of 1-*O*-hexadecenylglycerol ($C_{16:1}$) and 1-*O*-octadecenylglycerol ($C_{18:1}$), the main constituents of the glycerol ether fraction F4; percentages given by gas chromatography.

tions in the alkyl chains of the shark liver oil ether lipids have been determined recently in our laboratory by GC/MS, using and comparing two different approaches to fix the position of the ethylenic bond. First, a classical procedure was used, namely, the preparation of methoxy ether derivatives at the ethylenic have position of the alkylchain (40); second, an original technique, the use of soft chemical ionization with nitric oxide as reagent gas, developed by Malosse and Einhorn (41) for straight long-chain unsaturated olefins, alcohols, acetates, and aldehydes, was also performed satisfactorily on monounsaturated 1-*O*-alkylglycerols. These results will be published elsewhere.

*The use of urea complexation to produce substantial amounts of pure 1-*O*-monoalkyl and monoalkenylglycerols.* From the data of our urea complexation experiments, it is possible to imagine an efficient purification procedure as follows. Step 1: urea complexation of the initial fraction containing both saturated and monounsaturated GE with a high ratio of urea to GE (*R*), which will lead to the recovery of a pool of pure monounsaturated GE in the filtrate, but with a poor yield (40%); the yield can of course be improved by reprocessing the precipitate. Step 2: urea complexation of the monounsaturated GE fraction with a low *R*, to separate the GE as a function of the carbon chain length.

This procedure will be reduced to step 2 if only long-chain monounsaturated GE are of interest, and this makes urea complexation a powerful means for removal of the longer-chain monounsaturated ethers from a mixture of saturated and monounsaturated glycerol ethers. This novel use of the urea complexation method can provide useful amounts of pure 1-*O*-monoalkylglycerols. These compounds could be utilized as precursors in the large-scale synthesis of a series of molecules with important potential uses in cosmetic as well as pharmaceutical areas.

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Microscale Preparation of Natural and Labeled Oxysterols

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ABSTRACT: The preparation of cholesterol and radiocholesterol oxidation products on a microscale is difficult. Cholesterol generally resists oxidation unless it is well dispersed under controlled conditions. A method was developed to reliably produce 7α- and 7β-hydroxycholesterol and 7-ketocholesterol. Small changes in pH, metal ions present, or in the colloidal dispersion, resulted in production of completely different oxysterols. Attempts to oxidize aged radiocholesterol were not successful even after purification by several thin-layer chromatographic steps, and this appeared to be due to a time-related change in the radioactive material. Fresh radiocholesterol oxidized readily.

Lipids 31, 529–534 (1996).

Recent intense interest in cholesterol and lipoprotein oxidation products prompted us to prepare oxysterols from cholesterol tracers labeled with carbon-14, carbon-13, and deuterium. The oxidation of cholesterol has been extensively studied and comprehensively documented (1). Our aim was to oxidize cholesterol in a way such as to yield a limited number of major products by a method that would give reasonable reproducibility. The method was based on that described by Bergstrom and Wintersteiner (2), in which air is bubbled through a heated colloidal dispersion of cholesterol. We chose this method because it yields the oxysterols 7α- and 7β-hydroxycholesterol and 7-ketocholesterol as its major products, leaving some unaltered cholesterol, which suited our purpose. Furthermore, the additional oxygen functions are introduced at the C-7 position where they would not interfere with the isotopic substitutions we intended to use. The method as originally described was for oxidizing 5.0 g cholesterol and it can be scaled up easily to process several times that quantity (3,4). However, it was not possible simply to scale down the method to oxidize milligram to microgram amounts of cholesterol because the reaction was not reproducible with respect to the yield of oxysterols. Furthermore, it was open to the atmosphere and soap bubbles tended to escape from the top of the reaction vessel during bubbling of oxygen, which was clearly unsuitable for oxidizing radioisotopes. To overcome these difficulties we devised a closed system in which cholesterol was uniformly distributed and oxygen bubbling was unnecessary. In this system yields of oxysterols were highly reproducible.

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Abbreviations: GC/MS, gas chromatography/mass spectrometry; TIC, total ionization current; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Materials. Cholesterol (Sigma grade), sodium stearate, and methoxyamine hydrochloride were purchased from Sigma (St. Louis, MO). 4-^{14}C-Cholesterol was purchased from Amersham (CFA.128, 55 mCi/mMol; Arlington Heights, IL) and from Dupont New England Nuclear (NEC-018, 51 mCi/mMol; Boston, MA). Aged lots were purchased 5–6 yr before use and kept at −15°C in their original containers in toluene. Fresh 4-^{14}C-cholesterol was used within three weeks of synthesis and was supplied in ethanol solution and stored at −15°C. Stable isotopic cholesterols—[26,26,26,27,27,27-^{2}H]cholesterol and [23,24,25,26,27-^{13}C]cholesterol—have been characterized previously (5). The ^{13}C-cholesterol tracer also contained 26% [23,24,25-^{13}C]-[26,27]*bis*-norcholesterol. Authentic oxysterols were purchased from Steraloids (Wilton, NH) and Sigma. Regisil RC-3 [*bis*(trimethylsilyl) trifluoroacetamide + 10% trimethylchlorosilane] was purchased from Regis (Morton Grove, IL). Thinlayer chromatography (TLC) microplates, 2.5 × 7.5 cm, coated with silica gel 60, were purchased from Alltech (Deerfield, IL).

Milligram scale. The following procedure is used to oxidize 40 mg of cholesterol: 8.0 mg of sodium stearate is introduced into a test tube and to this is added 8.0 mL water with pH adjusted to 8.5–9.5 by adding a few μL of 10% aqueous KOH, heated to 85°C and vortexed to bring the stearate into solution. This solution is not stable and is best if freshly prepared and kept at 85°C until used. The cholesterol is dissolved in 2.0 mL ethanol at 60–65°C and is added slowly in a fine stream to the stearate solution through a Pasteur pipette with a finely drawn-out tip while the solution is being vortexed. The colloidal preparation is brought to 85°C and transferred to a preheated 50-mL conical flask with a long neck stabilized on a heater with magnetic stirrer. It is necessary for the contents to be well stirred throughout and for the temperature to be maintained at 83–85°C. Oxygen is bubbled gently through the solution for six hours *via* a pipette with a fine tip.

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The soap bubbles and vapors rising in the flask are condensed on strips of aluminum foil hanging into the opening of the flask. The contents are allowed to cool, aliquots transferred to test tubes, and extracted three times with two volumes of ethyl ether. The ether is evaporated with heat in a stream of nitrogen. The products in ethanol are monitored by TLC on microplates eluted with ethyl acetate/heptane (3:1 or 3:2,vol/vol).

The constituents are visualized (6) by briefly dipping the plate, freed of solvent, in a solution of 10% $CuSO_4$ in 8.5% H_3PO_4 and heating it on a Pyrex dish on a hot plate. On heating, cholesterol appears as a magenta color, the 7-OH compounds (and others) are Prussian blue, and the 7-ketocholesterol becomes yellow/ocher after a delay. Charring depends on time and temperature. The compounds are identified by running suitable reference compounds in the same way and are verified by gas chromatography/mass spectrometry (GC/MS). The proportion of cholesterol oxidized in this system varied substantially and oxidation sometimes failed. This seems to be due to early destabilization of the sol recognized by the deposition of solids on the side of the flask.

Microgram scale. Because this system is not desirable for oxidizing 4-^{14}C-cholesterol due to the danger of spillage with bubbling oxygen and lack of reproducible yield, a new closed system was developed. Some of the variations tried in this system are described in detail to highlight points of interest.

System A. System A consists of 200 µg cholesterol plus 10 µL ethanol plus 40 µL of sodium stearate preparation (8.0 mg in 8.0 mL water at pH 8.5–9.5). This is simply a scaled-down reaction according to Bergstrom and Wintersteiner (2), except that no oxygen was bubbled through the reactants.

System B. System B is 200 µg cholesterol plus 100 µL ethanol plus 40 µL of sodium stearate preparation plus 360 µL of water at pH 8.5–9.5. (This is the method of choice for preparing the desired oxysterols.) In this system the concentration of cholesterol is reduced to 0.4 mg/mL from 4 mg/mL by the method of Bergstrom and Wintersteiner (2), but the ratios of cholesterol/stearate and ethanol/water are unchanged.

System C. System C contains 200 µg cholesterol plus 100 µL ethanol plus 40 µL of sodium stearate preparation. The ratio of ethanol/water is increased 3.6-fold over System B. This was done to examine whether the ratio of ethanol/water was critical to yield the desired products.

System D. This system has 200 µg cholesterol plus 100 µL ethanol plus 50 µL of 30 µM $CuCl_2$ in water to give a final concentration of 10 µM. Here copper is added as an oxidant to determine whether it would increase the yield of the desired oxysterols.

Oxidation is carried out in 2-mL thick-walled glass v-vials with Teflon-lined screw caps. The cholesterol is added to the vial from a stock solution in ethanol and the solvent evaporated with heat under a stream of nitrogen. The volumes of ethanol given previously are added, the vial capped, and it is vital to ensure that all the cholesterol is brought into solution using heat. The vial is brought to ambient temperature and centrifuged to bring down droplets. The heated aqueous material is then added, the vial immediately capped, vortexed, and heated at 93–95°C for the next 18–20 h. An hour after heating has commenced the vials are cooled, centrifuged, the air displaced with oxygen and returned to the heating block. This is done three times in the first 8–12 h and the vials are vortexed every few hours over the full period. The preparation is then dried by lyophilization in a Speed-Vac (Savant Instruments, Farmingdale, NY) and taken up in ethanol. Stearic acid can be removed by TLC, applying the sample to a microplate in which the bottom 16 mm has been briefly dipped in a solution of 10% aqueous KOH/ethanol (1:9, vol/vol) and the plate well dried prior to chromatography. If the plate is not treated with KOH/ethanol, stearic acid migrates as a distinct band near natural cholesterol and separates well from oxysterols.

GC/MS. For derivatization of samples for GC/MS, a few µg of the dry sample is heated in Teflon-lined screw-capped glass vials for 2 h at 80°C in 0.5 mL dry pyridine with methoxyamine hydrochloride (20 mg/mL), and then at 65°C for 30 min after adding 1.0 mL of 1/1 Regisil RC-3 in dry pyridine. Sterols are chromatographed on a 15 m × 0.25 mm DB-210 capillary column (J&W Scientific Folsom, CA) at 255°C and analyzed with a Hewlett-Packard (Wilmington, DE) 5970 electron impact ionization mass spectrometer (Fig. 1). Cholesterol, 7α- and 7β-hydroxycholesterol, and 7-ketocholesterol had principal ions of *m/z* 368, 456, 456, and 470, respectively. The 7-OH compounds have the same masses, but are widely separated on GC and easily distinguished. The appropriate reference compounds gave the same findings. Three small peaks appearing between 4.5 and 4.9 min on the total ionization current tracing (TIC) (Fig. 1, top line) each accounted for less than 1% of the TIC area. Oxidized hexadeuterated cholesterol was separated on a 2 mm × 137 cm column packed with 3% OV-17 on gaschrom Q (Applied Science Laboratories Inc., State College, PA) at 250°C with a 30 mL/min helium flow rate and admitted to a Finnigan (Sunnyvale, CA) 3300 mass spectrometer. The deuterated and ^{13}C-enriched sterols had principal ions higher by 6 and 5 mass units, respectively, with the same retention times as the natural compounds.

The yield and reproducibility of System B was assessed by oxidizing 200 µg quantities of natural cholesterol. This was dried and taken up in ethanol, a 50 µg aliquot was taken, and 10 µg 5α-cholestane (Sigma) in toluene was added as an internal standard. The mixture was derivatized as described previously, and analyzed in duplicate by GC with a flame-ionization detector using a 60 m × 0.75 mm glass capillary coated with 1µ SPB-1 at 310°C (Supelco Inc., Bellefonte, PA). The areas of the oxysterols, cholesterol, and 5α-cholestane were proportional to the mass present.

RESULTS

The development of a closed oxidation system for microgram quantities was undertaken for use with radioactive cholesterol. Unlike the open milligram-scale system, the closed microgram-scale system consistently gave the desired products using either natural cholesterol, deuterated cholesterol, ^{13}C-cholesterol, or fresh 4-^{14}C-cholesterol. An additional advan-

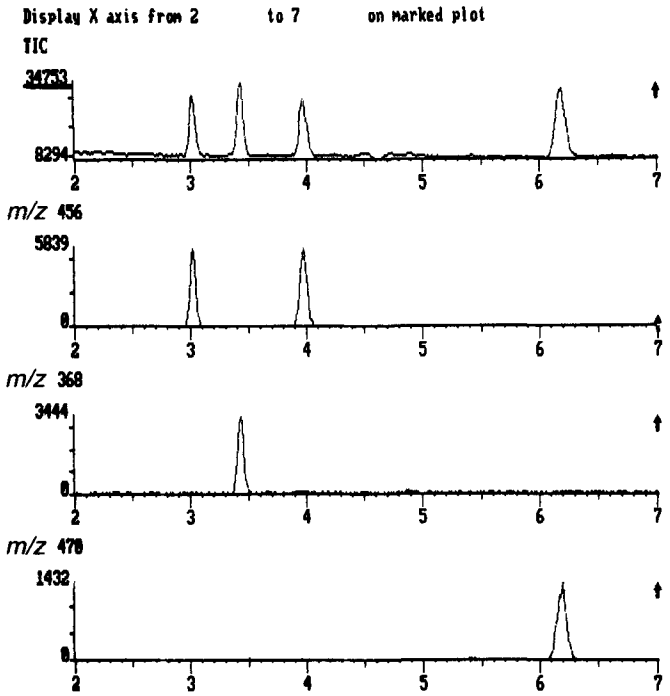


FIG. 1. Gas chromatography/mass spectrometry (GC/MS) of oxidized cholesterol. Natural cholesterol oxidized by System B was analyzed by capillary column GC/MS as described in the Materials and Methods section with monitoring of ions between *m/z* 250–600. The total ionization current (TIC) (top line), *m/z* 456 (second line, 7α- followed by 7β-hydroxycholesterol), *m/z* 368 (third line, cholesterol), and *m/z* 470 (bottom line, 7-ketocholesterol) are shown.

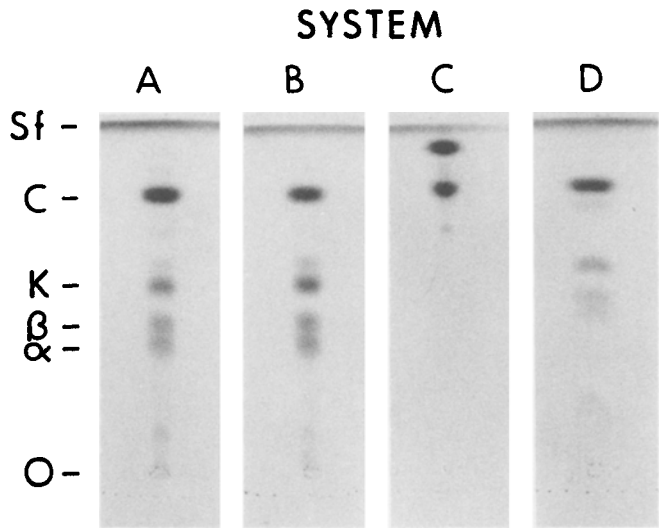


FIG. 2. Thin-layer chromatography of cholesterol and its products after oxidation in Systems A–D (see text for details). O is origin, α is 7α-hydroxycholesterol, β is 7β-hydroxycholesterol, K is 7-ketocholesterol, C is cholesterol, and Sf is solvent front.

tage is that it need not be closely monitored. Figure 2 shows charred TLC plates of cholesterol oxidized in Systems A–D. Systems A and B give much the same results with principal products of 7α- and 7β-hydroxycholesterol and 7-ketocholesterol. System C, which differs from A and B in having a higher proportion of ethanol to water, yielded a large amount of a compound less polar than cholesterol. System D, which included copper as an oxidant, produced little 7α- or 7β-hydroxycholesterol, but did produce 7-ketocholesterol identified by GC/MS. The differences between Systems A and B compared with Systems C and D (Fig. 2) might depend on the colloidal dispersion achieved. Preparations A and B were different in that A was densely opalescent and B very faintly so, although both remained homogeneous. We found System B most suitable for our purposes. The o.d. of System B at 600 nm standardized against water was 0.062, and was 0.056 to 0.058 at the end of treatment. This system can be adjusted to treat more or less starting material and one can easily cope with a considerable number of vials at the same time. In Systems C and D there is a precipitate. These preparations become homogeneous on heating, but the precipitates reform on cooling after oxidation and there is a separation into two phases. The physical dispersion of cholesterol thus differs considerably in these systems.

The method of extracting the sterols unexpectedly proved to be critical. For example, if 200 μg of cholesterol is oxidized by System B and, instead of drying, chloroform and ethanol are added in proportions to give a Folch-type of phase split (7), and a few μL of 2N HCl is added to this to promote phase separation, the phases separate readily with the aqueous phase at pH 2.0. However, the oxysterols recovered in the chloroform phase are substantially altered qualitatively (Fig. 3). Numerous new compounds are generated while others dis-

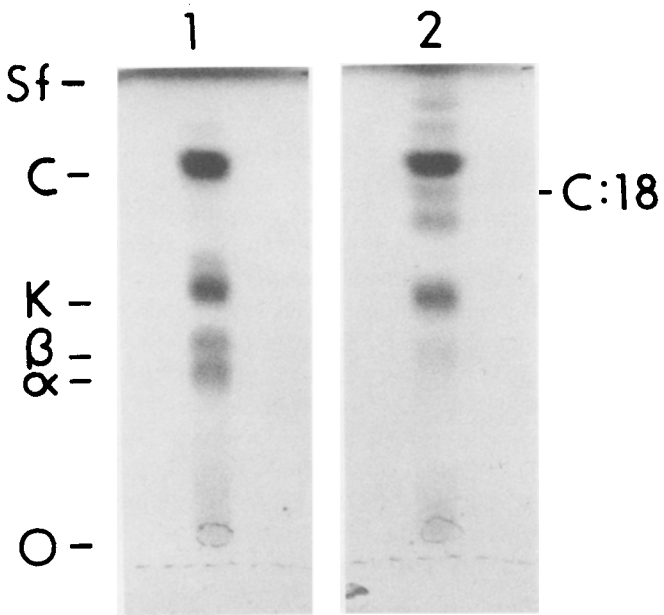


FIG. 3. Effect of acidification of solvent extract. Compounds are as named in Figure 2 and C:18 is stearic acid. Plate 1 is standard extraction procedure and plate 2 is an aliquot of the same preparation in which the solvent was acidified to promote a phase split (see text for details).

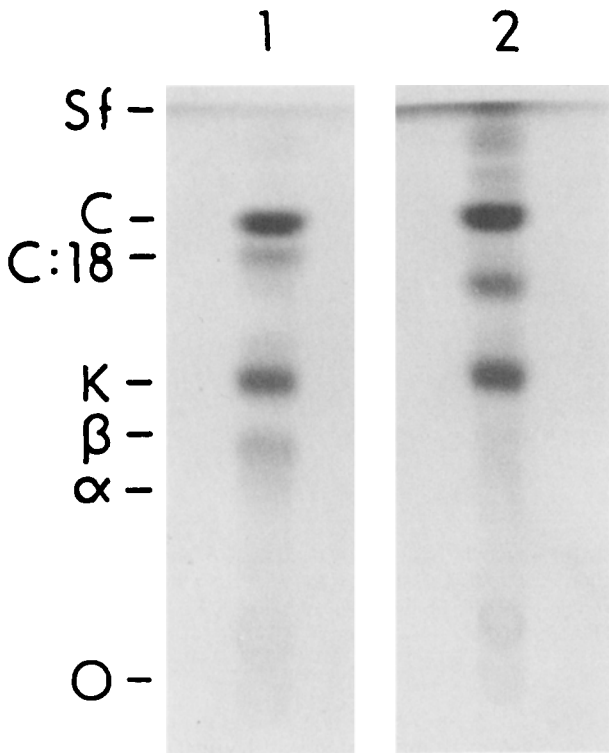


FIG. 4. Effect of alkali wash of solvent extract. Plate 1 is standard extraction procedure and plate 2 is after an aliquot was washed with potassium hydroxide as described in the text. Compounds identified as in Figures 2 and 3.

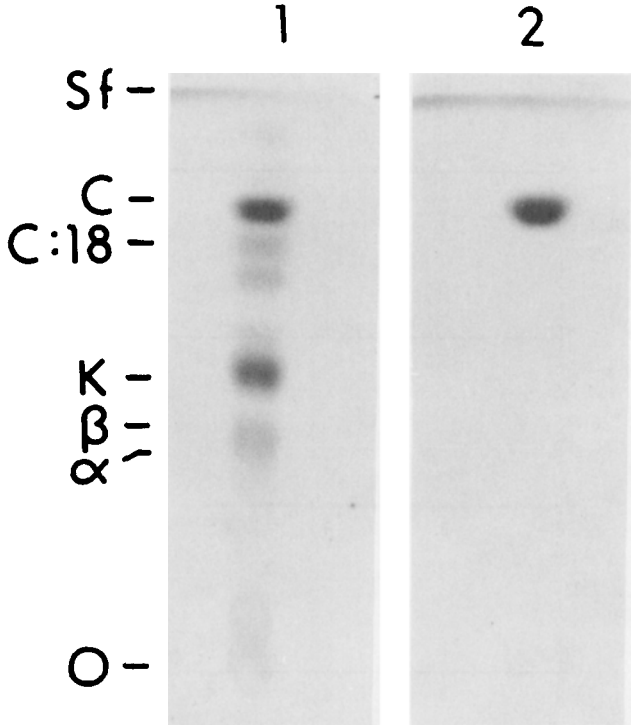


FIG. 5. Effect of bubbling oxygen through a colloidal dispersion of cholesterol. Plate 1 is standard microgram-scale oxidation method, whereas plate 2 shows recovery of unaltered cholesterol after bubbling of oxygen for 6 h at 85°C. Compounds identified as in Figure 2.

appear. If a similar experiment is done except that no acid is added and an alkaline wash of the organic phase is done to remove the stearate (2), then the nature of the oxidation products is also altered (Fig. 4). Bascoul *et al.* (8) appear to have made a similar observation with alkali, but they gave no details.

The effect of mild acid used to promote a phase split in the extraction of the oxysterols generated in System B was studied in more detail by GC/MS using a capillary column. The use of mild acid at pH 2.0 resulted in further oxidation of the oxysterols. It was found that 7α-hydroxycholesterol had disappeared, 7β-hydroxycholesterol was greatly reduced, and 7-ketocholesterol remained relatively abundant. Two principal new peaks appeared. One was identified as cholesta-3,5-diene-7-one by its retention time and a molecular ion of *m/z* 411 (methoxyamine derivative) and a major fragment at *m/z* 380 (411-OCH_3), identical to an authentic standard. The other new compound was derived from 7α-hydroxycholesterol, as demonstrated from reference material. It had prominent ions at *m/z* 412 and 456. The latter is the major fragment ion of the 7-hydroxycholesterols, suggesting that the new compound is a further oxidation product of those oxysterols.

The need for appropriate conditions in order to oxidize cholesterol is illustrated by the following experiment in which the cholesterol dispersion was destabilized by vigorously bubbling oxygen. Forty μL of sodium stearate preparation was added to 200 μg of cholesterol in 2.0 mL ethanol, and to this mixture 8.0 mL water at pH 8.5–9.5 was added. The material was then heated to 85°C for 6 h as oxygen was bubbled vigorously through a fine capillary. The recovered material (Fig. 5) was completely unchanged. The reason for resistance to oxidation is presumably destabilization of the solution as small particles appeared to precipitate from what had been a homogeneous solution and the o.d. of this dilute dispersion declined from 0.011 to 0.002.

Attempts to oxidize aged ^{14}C-cholesterol by the microscale method, with the mass of material calculated from the specific activity, failed repeatedly. The radiocholesterol was purified on silicic acid-coated plates in petroleum ether/ethyl ether/acetic acid (90:10:1, by vol) and also in antioxidant-free ethyl acetate/heptane (3:1 and 3:2, vol/vol) with over 87% of the radioactivity recovered in the position of cholesterol. An autoradiograph of the ^{14}C-cholesterol after purification is shown in Figure 6. The adjacent figure is an autoradiograph of the same material after attempted oxidation in System B. The yield of new compounds was barely detectable in numerous attempts at oxidation.

Figure 7 shows an autoradiograph of a preparation of fresh 4-^{14}C-cholesterol oxidized in System B, which has yielded the desired products in about the same proportions as found in simultaneously oxidized natural cholesterol. The distribution of radioactivity, obtained from scrapings taken from the TLC plate, was cholesterol, 45%; 7-ketocholesterol, 33.1%;

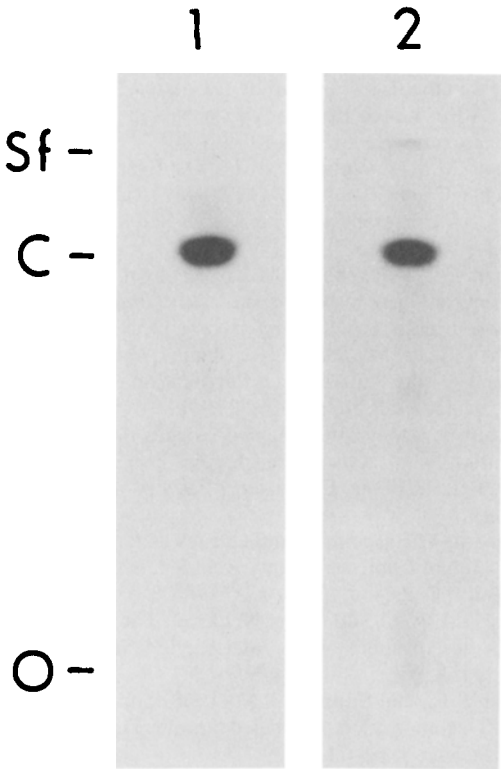


FIG. 6. Failure to oxidize purified aged ^{14}C-cholesterol. Autoradiographs of thin-layer chromatographic separations of purified "old" 4-^{14}C-cholesterol (plate 1) and of the same material after oxidation (plate 2), the latter showing the generation of negligible amounts of a number of new compounds. Compounds identified as in Figures 2 and 3.

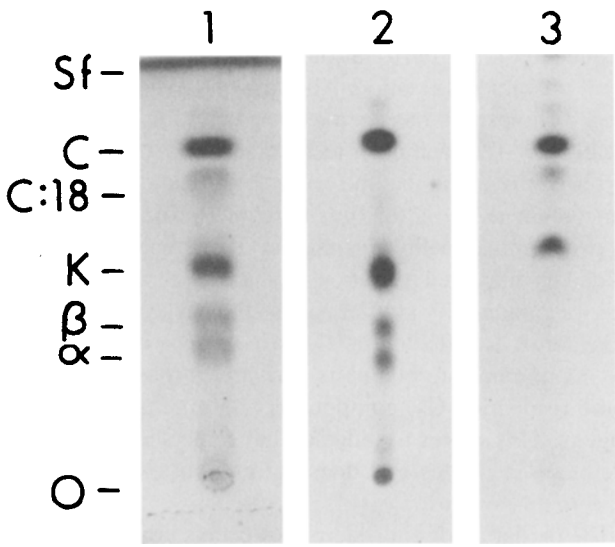


FIG. 7. Oxidation of fresh ^{14}C-cholesterol. From left to right: (1) thin-layer chromatographic (TLC) separation of natural cholesterol and its oxysterols treated by the microgram-scale method, (2) autoradiograph of a TLC separation of fresh 4-^{14}C-cholesterol treated by the microgram-scale method and extracted as recommended, and (3) autoradiograph of a TLC separation of material in 2 in which the sterols had been extracted with solvents that had been acidified (see text for details). Compounds identified as in Figures 2 and 3.

7β-hydroxycholesterol, 9.4%; and 7α-hydroxycholesterol, 12.5%. The effect of acidification of the solvent extract prior to recovery of the radioactive material is shown in the adjacent autoradiograph, again demonstrating changes similar to those found with natural cholesterol (see Fig. 3). It should be noted that the fresh 4-^{14}C-cholesterol had not been run on TLC prior to its oxidation.

The yield of oxysterols using the closed System B was quantitated by GC using a flame-ionization detector. In three experiments analyzed in duplicate the reaction products were cholesterol, 45.3 ± 1.8% (SE); 7α-hydroxycholesterol, 11.6 ± 0.5%; 7β-hydroxycholesterol, 13.6 ± 0.6%; and 7-ketocholesterol, 29.8 ± 0.8%.

DISCUSSION

The oxidation of free cholesterol is recognized to present problems in its execution (8,9). However, the method of Bergstrom and Wintersteiner (2), described decades ago, is highly effective and adaptable, once one recognizes those aspects that are vital for its success. With this method the creation of a colloidal dispersion of cholesterol and its maintenance are crucial for oxidation. Oxidation was prevented when the dispersion was disrupted by vigorously bubbling oxygen through the reaction mixture (Fig. 5). The conditions under which cholesterol is dispersed clearly affects the nature of the oxidation products (Fig. 2), as does altering the pH of the oxidized preparation during lipid extraction (Figs. 3, 4, and 7). There is obviously the potential for producing a wide range of compounds, probably many more than is shown here, both by modifying the nature of the colloidal dispersion, as well as by altering the pH of the final extract.

In contrast to the difficulty and variability of achieving controlled cholesterol oxidation for small samples by existing techniques, our preferred microscale method (System B), which is closed to the atmosphere, is highly consistent, both with respect to the products formed (Fig. 1) and their amounts (coefficient of variation 3.5–5.8% for percentage of each compound). To achieve this reproducibility, however, one must note the caveats given in the Methods and Materials section.

The most intriguing and taxing aspect of the work was the failure to oxidize aged ^{14}C- cholesterol, despite repeated purifications, whereas fresh material oxidized readily. The reason for that failure is by no means clear. It has been demonstrated that stored, labeled steroids are far from stable and some factors influencing stability have been identified (10,11). Purification of stored material at fairly frequent intervals seems to be obligatory for preventing breakdown and the presence of certain tars in the solvent are also deleterious.

TLC is commonly used to ensure purity and chemical

identity of labeled compounds. The failure to oxidize cholesterol that had been stored for some years under the conditions in general use leaves one uncertain about both points. Conceivably this was because the material was no longer cholesterol or that there persisted impurities, or that impurities were introduced during purification and/or recovery procedures, which prevented oxidation. Aberrant physiological behavior of aged cholesterol has been reported previously despite purification by TLC and other techniques (12).

It is worth noting that the original ^{13}C-cholesterol preparation contained 26% [26,27]*bis*-norcholesterol, which is missing two terminal methylene groups. Oxidation of this mixture yielded the expected oxidation products of cholesterol and, in addition, apparently similar oxidized derivatives of *bis*-norcholesterol, as reflected in TLC separations of the mixture. The *bis*-norcholesterol and its derivatives run slightly ahead of the respective C_{27} compounds and give the same chromogens. This serves to indicate that some shortening of the side chain of cholesterol does not prevent oxidation of the sterol in this system.

The oxidation of cholesterol has been studied extensively in a variety of systems (1). These include prolonged exposure of free cholesterol in the dry state at high temperature (9,13), the use of esters (8,9), and the dispersion of cholesterol in aqueous medium (9,14–16) the latter dating from the work of Bergstrom and Wintersteiner (2). More than 50 oxidation products have been identified (1,17). The purpose of this communication is to describe a micromethod for the oxidation of cholesterol, as well as of radioactive cholesterol, which is both simple and highly reproducible with limited oxidation products. By controlling the dispersion of cholesterol in sodium stearate, the pH, and the method of extracting the product, it is possible to produce selected cholesterol autoxidation products useful in biological studies. This will allow physiological studies of tracer oxysterols in intact animals and cell culture models.

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An Improved Method for Rapid Analysis of the Fatty Acids of Glycerolipids

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ABSTRACT: An improved rapid procedure to determine the fatty acid composition of glycerolipids is described. The procedure includes KOH-catalyzed transesterification and high-speed gas chromatography. Glycerolipids (20–40 mg) were mixed with 2 mL of hexane and 0.2 mL of 2 M methanolic KOH at room temperature for 1–2 min. The fatty acid methyl esters in the hexane layer were analyzed by gas chromatography on 10% SP-2340 at 240°C. Methyl linolenate and docosahexaenoate eluted within 2 and 5 min, respectively. Analysis was thus completed within 5 min for common vegetable oils and 8 min for fish oils.

Lipids 31, 535–539 (1996).

The fatty acid (FA) composition of fats and oils is determined by gas chromatography (GC) as fatty acid methyl esters (FAME). FAME are usually prepared by (i) saponification of glycerolipids followed by methylation, (ii) acid-catalyzed methanolysis, or (iii) alkali-catalyzed methanolysis. Of the many procedures that have been proposed and used for FAME preparation (1–5), the KOH-catalyzed methanolysis (transesterification) has the great advantage of rapid and simple preparation (6–11). Nevertheless, this preparative method is not popular, probably due to researchers' apprehensions that glycerolipids may be saponified with KOH even in methanol to form free FA. In the most conventional methods of alkali- and acid-catalyzed methanolysis (1–5), lipid samples are refluxed with CH_3ONa in methanol and with anhydrous HCl in methanol, respectively, which are time-consuming procedures.

In 1969, Christopherson and Glass (12) reported an alkali-catalyzed methanolysis method and subsequent GC analysis for milk fat. In the present study, we thoroughly verified that the KOH-catalyzed methanolysis does not produce appreciable amounts of free FA and is superior to other methods in terms of rapidity, safety, and cost. We suggest the use of this convenient methanolysis as a superior method for preparation of FAME from glycerolipids. The combination of this method and high-speed GC has enabled the rapid analysis of FA compositions of various fats and oils.

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Abbreviations: DEGS, diethylene glycol succinate polyester; DGDG, digalactosyldiacylglycerol; FA, fatty acid; FAME, fatty acid methyl ester; GC, gas chromatography; PC, phosphatidylcholine; TG, triacylglycerol; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Reagents. Triacylglycerol (TG) and oleyl oleate were purchased from Nu-Chek-Prep (Elysian, MN). FA, cholesteryl oleate, and digalactosyldiacylglycerol (DGDG) were from Serdary Research Laboratories (London, Canada). SP-2340 is the product of Supelco (Bellefonte, PA). Methanol, hexane, and KOH were of reagent grade and were used without further purification.

Preparation of FAME from glycerolipids. In a small glass tube were placed 20–40 mg of TG, 2 mL of hexane, and 0.2 mL of 2 M methanolic KOH. Plastic microcentrifuge tubes (1.5 mL) were also used in a scale of operation 20-fold smaller. In a typical reaction, the tube was vortexed at room temperature for 2 min or at 30–37°C for 1 min. An aliquot of the upper hexane layer was directly injected in to GC (Hitachi 263-70, a 2-m glass column packed with 10% SP-2340 on 100–120 mesh Chromosorb WAW). The column temperature was 240°C for high-speed GC.

The total FA composition of a lipid mixture was determined by a conventional saponification/methylation procedure. The lipid mixture (20–60 mg), contained in a screw-capped glass tube, was hydrolyzed with 2 mL of 0.5 M KOH in 70% methanol at 70°C for 1 h. After acidification with 0.6 mL of 2 M HCl, the FA released were extracted with hexane. The hexane solution was washed with water and then evaporated *in vacuo*. The free FA obtained were methylated with 15% methanolic BF_3 for 20 min at room temperature.

The reaction products were analyzed by thin-layer chromatography (TLC) on silica gel (No. 5721; E. Merck, Darmstadt, Germany). The solvent system used for methanolysis products of TG was hexane/acetone/acetic acid (95:5:0.5, by vol). The products of polar lipids were analyzed by double development; the first solvent was chloroform/methanol/water/acetic acid (55:45:7:0.5, by vol), and the second was hexane/acetone/acetic acid (95:5:0.5, by vol). Lipids separated were visualized by spraying 50% (w/w) sulfuric acid and then heating at 135°C.

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RESULTS AND DISCUSSION

Effects of the KOH concentration and the reaction time on FAME formation. The maximum conversion of TG into FAME was obtained at 2 M KOH (Fig. 1A), when the reaction mixture was vortexed at room temperature for 2 min (Fig. 1B). A trace amount of TG remained unchanged at room temperature at 1 M KOH, while no TG remained at 30–37°C (data not shown). The FA compositions obtained from these complete and incomplete methanolysis procedures were almost the same. Although vortexing for 2 min at room temperature or for 1 min at 30–37°C is recommended, vortexing for 1 min at room temperature or for 0.5 min at 30–37°C can also be used for more rapid analysis. It has been reported that mixing for 2 min is enough for methanolysis (12).

When TG in hexane was allowed to stand overnight with methanolic KOH at room temperature, using diluted methanolic KOH solutions (0.1–0.2 M) gave better results than using more concentrated solutions (0.5–2 M) (Fig. 1C). Appreciable amounts of free FA were detected at concentrations higher than 0.5 M, although no TG was found in the range of the concentrations tested. Another practical procedure that can be used in FAME preparation is to allow TG to stand for 2 h with 0.2 M KOH. It should be noted, especially for autosampler users, that the methanolysis of soybean oil with 2 M KOH for 24 h decreased the linoleic acid (18:2) and linolenic acid (18:3) methyl ester contents from 53.7 to 52.5% and from 7.9 to 7.3%, respectively, compared with the contents obtained by the 2-min reaction. NaOH can be replaced with KOH, but the former is less soluble in methanol than the latter. The methanolic KOH solutions can be stored in tightly capped plastic bottles at room temperature for at least two months, without any decrease in reactivity.

Effects of the water content of methanol on FAME formation. The water content of methanol at 2% or less did not affect FAME formation (Fig. 1D), and hence commercial methanol of reagent grade can be used without drying. When TG in hexane was allowed to stand overnight with 0.2 M methanolic KOH, even 10% water in methanol did not affect the methanolysis (data not shown). The reaction proceeded in the presence of silica gel and small amounts of water, and hence glycerolipids that were separated by TLC could be treated without extraction with solvents.

Effects of the amounts of methanolic KOH and TG on FAME formation. An amount of methanolic 2 M KOH solution in the range of 0.1–0.8 mL did not affect the reaction (Fig. 2A). In this reaction system, 200 mg of TG was completely converted into FAME (Fig. 2B). Thus, the amounts of both methanolic KOH and TG do not affect the reaction efficiency.

Specificity for ester linkage. DGDG and PC were also transesterified with methanolic KOH (Fig. 3A). Most of the PC molecules were converted into FAME within 5 s; after

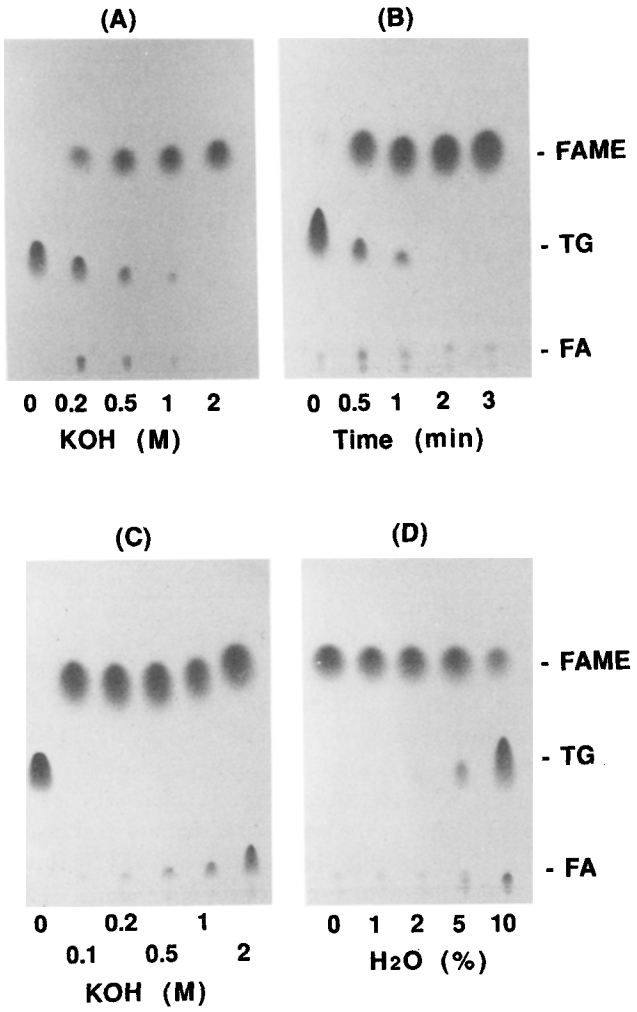


FIG. 1. (A) Dependence of the methanolysis of triacylglycerol (TG) on the KOH concentration. The reaction mixtures were vortexed for 2 min. (B) Time course of the methanolysis of TG. The KOH concentration was 2 M. (C) Dependence of the methanolysis of TG on the KOH concentration. The reaction mixtures were allowed to stand for 15 h. (D) Effects of water on methanolysis of TG. The KOH concentration was 2 M. FA, fatty acid; FAME, FA methyl esters.

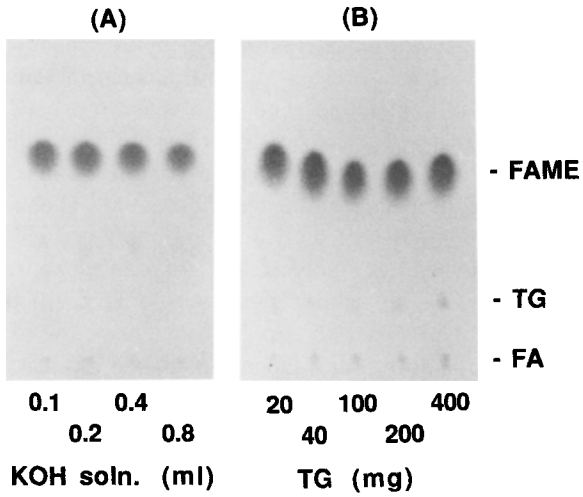


FIG. 2. (A) Dependence of the methanolysis of TG on the amount of methanolic 2 M KOH. (B) Effects of the amount of TG on methanolysis. Abbreviations as in Figure 1; soln., solution.

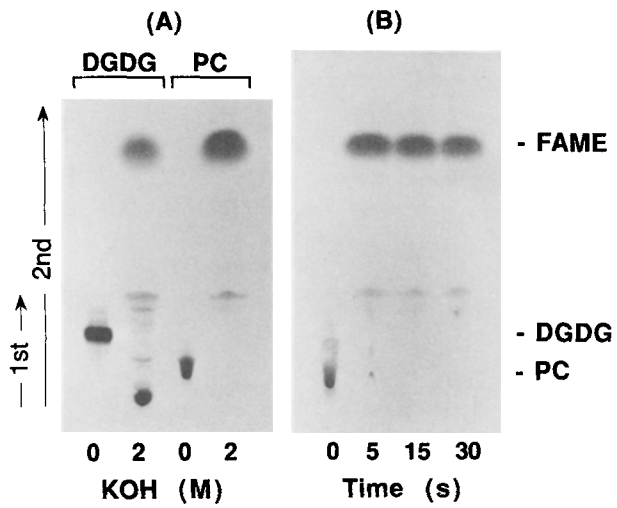


FIG. 3. (A) Methanolysis of digalactosyldiacylglycerol (DGDG) and phosphatidylcholine (PC). (B) Time course of the methanolysis of PC. After the termination of reaction by acidification with acetic acid, the solvents in the reaction mixture were evaporated *in vacuo*. The total residue was dissolved in chloroform/methanol (1:1, vol/vol) and double-developed on silica gel with two different solvent systems (see the Materials and Methods section). Other abbreviation as in Figure 1.

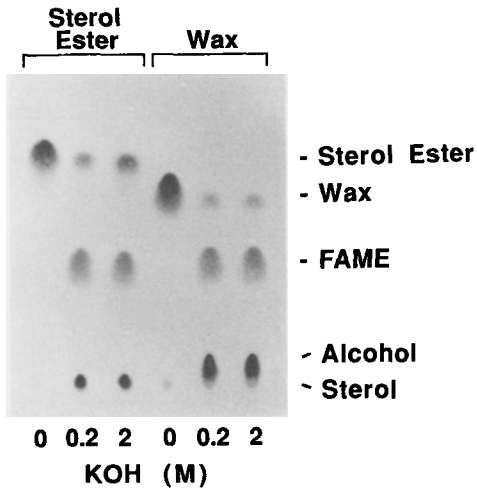


FIG. 4. Methanolysis of sterol ester (cholesteryl oleate) and wax (oleyl oleate). The reaction time was 15 h. The solvent system was hexane/benzene/acetic acid (50:50:0.5, by vol). Abbreviation as in Figure 1.

treatment for 15 s, no PC remained (Fig. 3B). The high reaction-rate for PC is probably due to its high solubility to methanol. In contrast to these glycerol esters, sterol ester and higher-alcohol ester (wax) did not react with methanolic KOH under the conditions described in the Materials and Methods section. Even when the hexane solutions of these inert esters were allowed to stand overnight with 0.2 or 2 M methanolic KOH, small amounts of the original esters remained unchanged (Fig. 4). Methanolysis of sterol esters requires the addition of benzene (40%) and a prolonged reaction time (2 h) (13).

Determination of the FA composition of glycerolipids. The present method was compared with two conventional methods, saponification/methylation and CH_3ONa-methanolysis with reflux, as to the FAME products (Table 1). The lipid sample was a mixture of TG (oleic acid, 18:1), PC (erucic acid, 22:1), and free FA (18:2). In spite of the presence of large amounts (37–60%) of free FA (18:2), only trace amounts of 18:2 were detected in FAME prepared by the KOH-catalyzed methanolysis. The mechanism by which the methyl linoleate was formed is not known. No appreciable difference was found between the present method and CH_3ONa-catalyzed methanolysis with reflux. Because free FA in fats and oils are not converted into methyl esters by alkali-catalyzed methanolysis, the FA composition obtained by the present method does not precisely reflect the total FA composition. However, free FA are a minor component of natural fats and oils (usually less than a few percent of TG), and the FA composition of free FA is similar to that of TG. The FA compositions determined for natural fats and oils by the present method are therefore expected to be almost the same as the total FA compositions. The FA composition of soybean oil was determined by the above three methods. The results were almost identical (Table 2). These data clearly

TABLE 1
Effects of Lipid Types on FAME Formation by Three Different Methods[a]

	FAME formed (%)								
	KOH saponification/BF_3 methylation[b]			KOH methanolysis[c]			CH_3ONa methanolysis[d]		
Lipid mixture	18:1 (TG)	22:1 (PC)	18:2 (FA[e])	18:1 (TG)	22:1 (PC)	18:2 (FA[e])	18:1 (TG)	22:1 (PC)	18:2 (FA[e])
TG + PC	58.5	41.5	—	58.5	41.5	—			
TG + FA[e]	49.9	—	50.1	99.6	—	0.4			
PC + FA[e]	—	40.5	59.5	—	99.6	0.4			
TG + PC + FA[e]	36.2	26.8	37.0	58.2	41.6	0.2	58.2	41.7	0.1

[a]The fatty acid (FA) components of triacylglycerol (TG), phosphatidylcholine (PC), and free FA were 18:1, 22:1, and 18:2, respectively. Each value is an average of two determinations. FAME, fatty acid methyl ester.
[b]Saponification with KOH in aqueous methanol, followed by extraction with hexane and by methylation in the presence of BF_3.
[c]KOH-catalyzed methanolysis.
[d]CH_3ONa-catalyzed methanolysis under reflux.
[e]Free FA.

TABLE 2
The Fatty Acid (FA) Composition of Soybean Oil[a]

Method	FA (%)				
	16:0	18:0	18:1	18:2	18:3
KOH saponification/BF_3 methylation[b]	10.6	3.8	23.1	53.7	8.9
KOH methanolysis[b]	10.5	3.7	23.3	53.7	8.7
CH_3/ONa methanolysis[b]	10.6	3.6	23.2	54.0	8.6

[a]Each value is an average of two determinations.
[b]See legend in Table 1.

show that the FA compositions of glycerolipids can be determined accurately by KOH-catalyzed methanolysis.

GC. The polyester liquid phase, diethylene glycol succinate polyester (DEGS), is one of the most widely used column-packings for FAME analysis. If DEGS is used to replace SP-2340 (used in this study), the KOH-catalyzed methanolysis should be terminated by adding 3 mL of 0.15 M acetic acid before GC analysis. If this is not done, DEGS will quickly deteriorate. DEGS is not suitable for high-speed GC because its maximum temperature of operation is 210°C.

SP-2340 is a more polar liquid phase than DEGS, and it is also more thermostable (up to 260°C) than DEGS. All of the common C_{18} saturated and unsaturated FAME were well separated from each other within 2 min at 240°C on 10% SP-2340 (Fig. 5A). Under the same conditions, the retention times of eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6) methyl ester prepared from a 22:6-enriched fish oil were 2.8 and 4.2 min, respectively (Fig. 5B). GC was also successfully carried out at 225°C with 5% SP-2340 on 80–100 mesh Chromosorb WAW. Retention times were shorter under the latter conditions than under the former ones, whereas separation was better under the former conditions than under the latter ones. The analytical values obtained under these two different conditions were almost the same (data not shown). The major disadvantage of SP-2340 is that hydroxy FAME, such as methyl ricinoleate, are eluted very slowly. The amounts of glycerolipids could also be determined using an internal standard.

Concluding remarks. Methanolysis that is conventionally carried out with reflux can be replaced by the KOH-catalyzed methanolysis without reflux. If free FA are not a major component of a lipid sample, HCl-catalyzed methanolysis, which converts both glycerolipids and free FA into FAME but requires heating for 1–2 h in sealed tubes, can also be replaced by this method. The only reagents used for the present procedure are KOH, hexane, and methanol. Harmful solvents such as benzene or chloroform are not used, and neither heating nor reflux is required. Only glass or plastic tubes are required, with no need for elaborate apparatus such as reflux condensers. Many lipid samples can be treated without difficulty. The data obtained are identical to those determined by other time-consuming methods. Thus, the FA composition of a lipid sample can be determined easily in a short time, e.g., 4–5 min for common vegetable oils and 7–8 min for fish oils, by the combination of KOH-catalyzed methanolysis and high-speed GC with SP-2340.

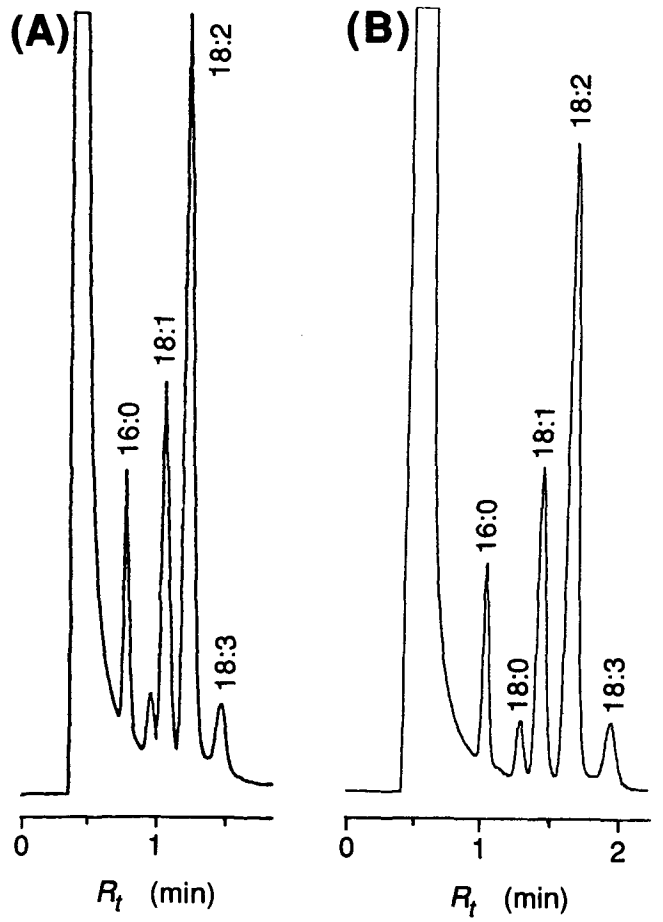


FIG. 5. High-speed gas chromatography (GC) of fatty acid methyl esters prepared from (A) soybean oil and (B) a 22:6-enriched fish oil. GC conditions: 3 mm x 2 m glass column; column packing, 10% SP-2340 on 100–120 mesh Chromosorb WAW; column temperature, 240°C; N_2, 40 mL/min.

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Synthesis of 1,2-Diacyl-*sn*-Glycerophosphatidylserine from Egg Phosphatidylcholine by Phosphoramidite Methodology

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ABSTRACT: A simple chemical method for the synthesis of 1,2-diacyl-*sn*-glycerophosphatidylserine (PS), with the same fatty acid composition in the *sn*-1 and *sn*-2 glycerol positions as egg phosphatidylcholine (PC), is described. PS synthesis was carried out by a phosphite–triester approach, using 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphorodiamidite (phosphoramiditate) as the phosphorylating agent, for the formation of phosphate linkage between serine and diacylglycerol. 1,2-Diacylglycerol, obtained from PC hydrolysis by phospholipase C, was coupled with *N-t*-BOC-L-serinebenzhydryl ester phosphoramidite with tetrazole as catalyst. Phosphite–triester was oxidized to the corresponding phosphate–triester with 30% H_2O_2 in CH_2Cl_2. The cyanoethyl group was removed by addition of an Et_3N/CH_3CN/pyridine mixture, and trifluoroacetic acid was used to eliminate the protecting groups of *O*-(1,2-diacylglycero-3-phospho)-*N-t*-BOC-serinebenzhydril ester. Purified PS was identified by thin-layer chromatography, infrared, and ^1H nuclear magnetic resonance.

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Phospholipids are major components of cell membranes, have many functions in living organisms, and are involved in a myriad of industrial processes (1). Phospholipids, particularly phosphatidylcholine (PC) and phosphatidylserine (PS), are used extensively in the formation of liposomes that have an enormous potential as drug delivery systems (2,3). Liposomes have also been used as model membrane systems to study cell–cell or liposome–cell interactions, as well as the molecular mechanisms of biological membrane fusion (4).

The isolation of phospholipids in pure and homogeneous form from natural sources or phospholipids mixtures, as well as the total synthesis of phospholipids by chemical reactions, is a complicated, expensive, and time-consuming task (5). Preparation of pure phospholipids, including PS, have been an area of interest because of their use in biochemical and membrane research. The conversion of phospholipids using synthetic methodology can be achieved both chemically and enzymatically. The economic efficiency of the chemical or biochemical process depends mainly upon high rates of production of desired products and minimizing the formation of undesirable by-products (6).

Although PS formation by enzymatic methods, using cabbage phospholipase D, has been reported by many scientists (7–9), the yields obtained were low and formation of the by-product phosphatidic acid was very high. Nevertheless, conversion of PC to PS by streptomyces phospholipase D was achieved in high yield (10).

PS with saturated or unsaturated acyl moieties also have been synthesized by a variety of chemical methods, most of which involve condensation of phosphatidic acid with serine protected at the amino and carboxyl functions or the coupling of diacylglyceroliodohydrines with suitable protected *O*-phosphoserines (5,11–14). In some cases, the amino and carboxyl protecting groups have been removed by two successive reactions leading to lower yield of the end product (12). Hermetter *et al.* (13) used the *N-tert*-butoxycarbonyl (*t*-BOC) residue and the benzhydryl group to protect serine at the amino and carboxyl groups, respectively. These two protective groups can easily be coupled to serine and removed in a single step under mild conditions, but the condensation yield of *N-t*-BOC-serine benzhydryl ester with defined molecular species of phosphatidic acids depends on the acyl chain lengths and their unsaturation degree (13).

We have studied the synthesis of PS starting from PC, the main constituent of natural phospholipids, using the phosphite–triester approach for the formation of a phosphate linkage between the serine and the diacylglycerol (DAG). Although phosphite coupling reactions have been well established and applied for oligonucleotides synthesis (15–19), the utilization of these tactics for the construction of phospholipids and their derivatives has received limited attention (20–25). Traditional phosphate coupling procedures and modifications thereof have generally provided a suitable mean for the preparation of phosphatidylethanolamine, PC, and deriva-

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Abbreviations: BE, *N-t*-BOC-L-serine benzhydril ester; BEP, *N-t*-BOC-L-serine benzhydril ester phosphoramidite; DAG, diacylglycerol; DDM, diazodiphenylmethane; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PS, phosphatidylserine; PS_P, *O*-(1,2-diacylglycero-3-phospho-)-*N-t*-BOC-L-serine benzhydril ester; *t*-BOC, *N-tert*-butoxycarbonyl; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

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tives bearing different fatty acid side chains and/or polar head groups, in general, with good to excellent yields. However, the application of these standard techniques to achieve an efficient synthesis of PS may be problematic; in fact, only single molecular species of PS have been synthesized by this technology.

The aim of this work was the synthesis of a PS with the same fatty acid composition in the *sn*-1 and *sn*-2 glycerol positions as egg PC used as the starting material. This will enable us to prepare liposomes with different PC/PS ratios but identical fatty acyl composition of each component, thus allowing us to correlate the changes in behavior of these model systems with the head polar group modification.

MATERIALS AND METHODS

Egg lecithin (65%) for synthesis was purchased from Merck A.G. (Darmstadt, Germany) and purified by column and thin-layer chromatography (TLC) before use. CH_3CN [Solvants Documentation Syntheses (SDS), Peypin, France; water content <50 ppm] was stored over CaH_2 (Merck A.G.). All other reagents were of analytical grade. Water was twice-distilled (Millipore Systems, Molsheim, France), and all organic solvents were redistilled before use.

DAG was obtained by PC hydrolysis with phospholipase C (26) (Lecithinase C; E.C. 3.1.4.3.) Type I of *Clostridium perfringens* (*C. welchii*, 10–20 units per mg protein; Sigma Chemical Co., St. Louis, MO). Phospholipase C (33 mg) dissolved in 60 mL of 0.1 M Tris-HCl buffer (pH 7.2) and 75 mL of a 5 mM $CaCl_2$ solution were added to a solution of PC (300 mg, 0.4 mmol) in peroxide free diethyl ether (75 mL). The mixture was stirred under N_2 at room temperature for 2 h and 45 min. 1,2-DAG_s were then extracted with diethyl ether (4 × 75 mL). The combined organic extracts were evaporated under reduced pressure, without heating, to reduce the initial volume four times. The resulting suspension was dried over anhydrous Na_2SO_4, the solvent was evaporated under reduced pressure to give a crude product, which was dissolved in dry CH_2Cl_2, and the drying and evaporation processes were repeated. This compound was used without further purification in the next step. The extent of the phosphocholine group elimination was checked by TLC, ^1H nuclear magnetic resonance (NMR), and infrared. DAG was used immediately in order to prevent isomerization from 1,2-DAG to 1,3-DAG .

Diazodiphenylmethane (DDM), often used as a carboxyl protecting group, was prepared from benzophenone hydrazone (>98%; Merck) by oxidation with metachloroperbenzoic acid (55%; Merck) in the presence of iodine as catalyst and *N,N,N′,N′*-tetramethylguanidine as base (Merck A.G.) (27). *N,N,N′,N′*-tetramethylguanidine (1.6 mL) and a solution of I_2 (1%, p/v) in 0.32 mL of distilled $CHCl_3$ were added to a solution of benzophenone hydrazone (1638.1 mg, 8.36 mmol) in 8 mL of distilled $CHCl_3$. After stirring for 5 min at 0°C, solid 55% metachloroperbenzoic acid (3409.8 mg, 10.87 mmol) was added slowly over 1 h at 0°C. The mixture was stirred for an additional 15 min, then washed with water until the washings were at pH 6. The resulting suspension was dried over anhydrous Na_2SO_4, the solvent was evaporated under reduced pressure and the residue suspended in MeOH. An assay at 526 nm (ε^{526}_{DDM} (MeOH) = 100 M^{-1} cm^{-1}) of a suitable diluted sample corresponded to a 90% yield of DDM.

Tetrazole (99%) and 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite (phosphoramiditate) were from Aldrich Chemical Co. Ltd. (Milwaukee, WI). The phosphoramiditate was checked by ^{31}P NMR (δ_P 123.5) and TLC (Table 1) before use. Impurities observed were 5.1% $(^iPr)_2NP(OCH_2CH_2CN)_2$ (δ_P 149.06), 1% $((^iPr)_2N)_2PO(CH_2CH_2CN)$ (δ_P 31.4), and phosphoramiditate hydrolysis products with low δ_P values.

N-t-BOC-L-serine benzhydril ester phosphoramidite (BEP) was obtained from *N-t*-BOC-L-serine benzhydril ester (BE). First, BE was prepared from *N-t*-BOC-L-serine (Sigma Chemical Co.) and an excess of DDM (13). DDM (938 mg, 4.83 mmol) was added to a solution of *N-t*-BOC-L-serine (717.7 mg, 3.5 mmol) in dry diethyl ether (20 mL), and additional diethyl ether was added to give a total volume of 30 mL. The reaction mixture was stirred overnight at room temperature. The excess of DDM was destroyed by the addition of glacial acetic acid and followed by the color change from red to yellow. The solvent was removed by evaporation under reduced pressure. The crude product was dissolved in $CHCl_3$ containing 1% per volume of aqueous NH_3 and purified afterwards by column chromatography on silica gel (27 g, 0.063–0.1 mm; Macherey Nagel, Düren, Germany). Elution was performed with chloroform containing 0.5% NH_3 to prevent decomposition of the product. Then 10-mL fractions were collected at a flow rate of 0.8–1.1 mL/mim. BE was contained in fractions 8–24. The combined fractions were filtered, dried, and evaporated under reduced pressure. BE (149 mg, 0.401 mmol) was dried by its dissolution in dry CH_3CN, and the solvent was evaporated under reduced pressure. Dry BE was redissolved under N_2 in dry CH_3CN (1.46 mL) by continuous stirring. Tetrazole

TABLE 1
R_F Values in Thin-Layer Chromatography

Compound[a]	Solvent systems[b] (1)	(2)	(3)
NBS	0.33 ± 0.03	—	—
BE	0.88 ± 0.02	0.25 ± 0.04	—
Phosphine	—	0.55 ± 0.02	0.56 ± 0.02
BEP	0.94 ± 0.03	0.42 ± 0.05	0.51 ± 0.07
DAG	0.87 ± 0.04	0.40 ± 0.05	0.46 ± 0.05
Phosphite triester	—	0.44 ± 0.06	0.47 ± 0.08
Phosphate triester	—	0.03 ± 0.02	—
PS_p	0.89 ± 0.04	—	—
PS	0.13 ± 0.02	—	—

[a]NBS, *N-t*-BOC-L-serine; BE, *N-t*-BOC-L-serine benzhydril ester; BEP, *N-t*-BOC-L-serine benzhydril ester phosphoramidite; DAG, diacylglycerol; PS_p, *O*-(1,2-diacylglycero-3-phospho-)-*N-t*-BOC-serine benzhydril ester; PS, phosphatidylserine.
[b]Solvent systems: (1) $CHCl_3$/MeOH/NH_3 (28%) (65:35:5, by vol); (2) hexane/diethyl ether/Et_3N (30:60:1, by vol); (3) hexane/diethyl ether (30:70, vol/vol). Adsorbent: Silica Gel G (Merck, Darmstadt, Germany). Data represent the mean ± SD of at least five determinations.

(26 mg, 0.37 mmol) was added with stirring at room temperature, followed by phosphoramiditate (120.4 mg, 0.4 mmol). The molar ratio of reagents was BE/phosphine/tetrazole (1:0.99:0.93). After stirring for 40 min, the reaction was stopped by the addition of 5% Et_3N in CH_2Cl_2. The diisopropylammonium tetrazolide formed was removed by washings with saturated aqueous NaCl and filtration. The solution was evaporated to dryness, and the residue was redissolved in CH_2Cl_2 and dried again with Na_2SO_4. The product, BEP, was identified by ^{31}P NMR and TLC on silica gel.

DAG (156.58 mg, 0.26 mmol) and BEP (265.9 mg crude BEP, 0.34 mmol pure BEP) were coevaporated in dry CH_3CN. The residue was dissolved in 1.65 mL of CH_3CN, and tetrazole (90.92 mg, 1.3 mmol) was then added. The reaction vessel was deaerated with N_2, and the mixture was stirred for 15 min (reagents molar ratio was DAG/BEP/tetrazole, 1:1.3:5). The reaction was stopped by the addition of 1.6 mL of 5% Et_3N in CH_2Cl_2, and the solution was washed in the same way as indicated for BEP. The amount of tetrazole necessary to activate the phosphoramidite was higher than that required to activate the phosphorodiamidite.

Subsequent oxidation of the intermediate phosphite delivered the corresponding phosphate–triester. The oxidation was carried out by the addition of 1.2–1.5 equivalent of an aqueous solution of H_2O_2 (30%) to the residue containing the phosphite–triester dissolved in 1.6 mL of CH_2Cl_2. The mixture was stirred vigorously at room temperature for two hours. The reaction mixture was washed with a saturated aqueous NaCl solution (2 × 1.6 mL), the layers were separated, and the combined organic phases were dried with anhydrous Na_2SO_4 and evaporated under reduced pressure. The phosphate–triester was deprotected without further purification.

Egg PC, and most of the naturally-occurring and biologically-active phospholipids, contain unsaturated *sn*-2-acyl chains possessing from one to four double bonds which can easily undergo oxidation. Thus, before the development of the synthetic method presented in this paper, we tested the H_2O_2 effect on fatty acid double bonds. We prepared an incubate containing a mixture of unsaturated fatty acids in CH_2Cl_2 and twice the theoretical amount of H_2O_2 necessary for the oxidation of the phosphite to the phosphate–triester. The mixture was stirred vigorously for two hours at room temperature and, after standard work up, fatty acids analysis by gas–liquid chromatography showed the same composition as the starting mixture (results not shown). Thus, no evidence of oxidation of fatty acyl double bonds in the presence of hydrogen peroxide used for phosphite oxidation was detected.

The cyanoethyl group was removed by addition of 1 mL of an Et_3N/CH_3CN/pyridine (1:1:1, by vol) mixture to the dry residue. After 90 min, the solvent mixture was eliminated by vacuum. The *O*-(1,2-diacylglycero-3-phospho)-*N*-*t*-BOC-serine benzhydril ester (PS_p) obtained was purified by column chromatography on silica gel (27 g, 0.1–0.2 mm; Macherey Nagel) saturated with $CHCl_3$ containing 0.5% NH_3 and eluting with $CHCl_3$ and $CHCl_3$/MeOH mixtures containing 0.1% NH_3. Then 5-mL fractions were collected at a flow rate of 0.7–1.0 mL/min. Pure PS_p was eluted when the $CHCl_3$/MeOH ratio was 90:10 (vol/vol). The combined fractions were filtered, dried, and evaporated under reduced pressure. trifluoroacetic acid (TFA) (1 mL) was added to the product dissolved in 1 mL of $CHCl_3$ to eliminate protecting groups of PS_p and was then removed by vacuum. Et_3N (0.4 mL), dissolved in $CHCl_3$, was added to the residue to neutralize any remaining TFA. The organic layer was washed with the following cold mixtures—MeOH/H_2O/$NaHCO_3$sat (2:1:1), MeOH/100 mM NaHCOO pH 3.0 (4:5), and MeOH/100 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid pH 7.4—to eliminate remaining Et_3N. The solvent was evaporated under reduced pressure and the product was dried over P_2O_5. The crude PS was purified by CM-Cellulose (CM52; Whatman, Kent, England) column chromatography using $CHCl_3$/MeOH mixtures as eluent, at a flow rate of 0.48 mL/min. Pure PS was eluted with $CHCl_3$/MeOH, 65:35 (vol/vol). The combined fractions were filtered, dried, and evaporated under reduced pressure.

Infrared spectra were obtained from a film of lipid solutions in chloroform (approximately 5 mg/mL) on a KCl pellet, using a Perkin-Elmer 687 infrared spectrophotometer (Norwalk, CT). All spectra were recorded at room temperature against a reference KCl pellet.

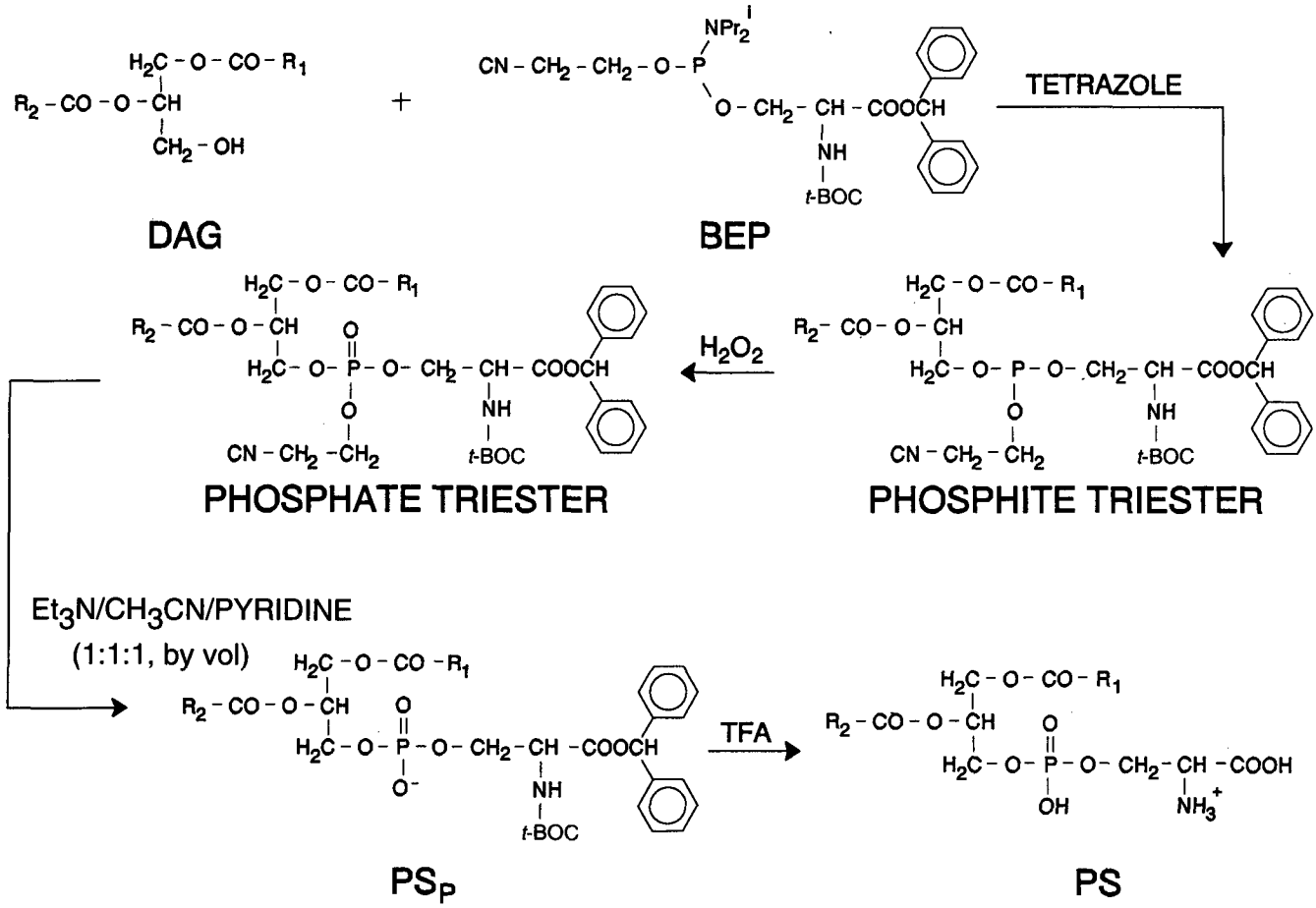
^1H NMR (200 MHz) and ^{31}P NMR (81 MHz) spectra were obtained, in $CDCl_3$ (15–25 mg lipid/mL), on Geminis-200 (Palo Alto, CA) and Varian XL-200 (Palo Alto, CA) spectrometers, respectively, operating in the Fourier transform mode, at room temperature. Typically 200–300 scans were averaged. Chemical shifts values are expressed in units of δ relative to tetramethylsilane set at 0 ppm or trimethyl phosphite set at 140–141 ppm, respectively, for ^1H and ^{31}P NMR spectra.

Methyl esters of the fatty acids were analyzed by gas–liquid chromatography on a Perkin-Elmer 990 gas chromatograph equipped with flame-ionization detector. Data were obtained with a 2 m × 2 mm i.d. column packed with 15% polymeric ethyleneglycol succinate as stationary phase on Chromosorb W (100–120 mesh; Johns-Manville, Denver, CO). Helium at 30 mL/min was used as carrier gas. Injector, column, and detector temperatures were 230, 188, and 250°C, respectively. Samples were applied as hexane solutions.

RESULTS AND DISCUSSION

Scheme 1 shows the scheme used for PS synthesis *via* phosphite coupling. ^{31}P NMR was used to check the reaction course.

DAG (98%) obtained by PC hydrolysis gave single spots on silica gel G plates using different solvent systems (Table 1), showing that the isomerization of 1,2 diglyceride to 1,3 diglyceride (side product) was not done during the reaction time course. Moreover, the extent of acyl migration was low (<4%) during the condensation step between DAG and BEP. The signal at δ_H 3.35 corresponding to $(CH_3)_3N^+$- from PC disappears for DAG, and the signals at δ_H 3.8–4.6



SCHEME 1

due to $-(CH_2O)_2P(O)O^-$ are not shown in DAG spectrum. On the other hand, the absorption bands at 970, 1090, and 1240 cm^{-1} related with the phosphate group are missing in DAG infrared spectrum.

BE was identified by TLC (Table 1), ultraviolet-spectrum ($CHCl_3$) (λ_{max}= 258 nm, ε = 450 $M^{-1}cm^{-1}$) and ^1H NMR (signals at δ_H 1.44 and δ_H 7.32–7.34 ppm corresponding to $-C(CH_3)_3$ and $-C_6H_5$ groups, respectively) (13).

The homogeneity of BEP, obtained from *N-t*-BOC-L-serine, was checked by TLC (Table 1). BEP ^{31}P NMR spectrum showed two main signals at δ_P 148.1 and 147.4 ppm. The two signals at similar δ_P obtained for BEP with different values showed the stereoselectivity of the reaction. The only impurities observed were little amounts of dimers (δ_P 137.8 ppm) and hydrolysis products (δ_P 2.1–18.5 ppm). The process was carried out with an excellent yield (95.5%), and BEP purity was *ca.* 71.7%.

The appearance in the ^{31}P NMR spectra of two new signals, after DAG and BEP condensation, at δ_P 136.37 and 135.85 ppm and the disappearance of the characteristic peaks of BEP isomers showed the formation of the desired phosphite–triester (93%). The oxidation of the phosphite to phosphate–triester was performed using a two-phase system with aqueous H_2O_2 and dichloromethane (21). This milder treatment was preferred over iodine oxidation (16) to avoid possible addition of iodine to fatty acid double bonds and because the oxidation solution is easy to remove from the phospholipid solution. The oxidation reaction was completed after two hours (94%) as confirmed by the substitution of phosphite–triester signals by two signals at δ_P –4.54 and –4.93 ppm in the ^{31}P NMR spectra.

The elimination of the cyanoethyl group and the purification of the obtained PS_P by column chromatography on silica gel was achieved by a 50% yield. BEP, phosphite–triester, and phosphate–triester were not extensively purified since the side products, largely consisting of hydrolysis products, did not interfere with the coupling, oxidation, and cyanoethyl group elimination processes.

PS obtained after deprotection with TFA was purified by CM-Cellulose (51%) column chromatography because the utilization of silica gel always gave impure PS. CM-Cellulose was used in older literature to separate PS and phosphatidic acid (8), although the basis for lipid separation on CM-Cellulose is not completely understood. It seems likely that in chloroform, which has a low dielectric constant, phospholipids are bound to CM-Cellulose by nonspecific adsorp-

TABLE 2
Fatty Acids Molar Content (%) Determined by Gas Chromatography[a]

Lipid species	Total fatty acids[b] (mol%)						
	16:0	16:1	18:0	18:1	18:2	20:4	22:6
PC	35.3 ± 0.7	1.1 ± 0.1	11.3 ± 0.8	30.0 ± 0.3	18.4 ± 1.0	3.3 ± 0.1	0.5 ± 0.1
DAG	36.1 ± 1.4	0.9 ± 0.1	12.1 ± 0.7	29.7 ± 1.2	17.5 ± 1.3	3.2 ± 0.1	0.5 ± 0.1
PS_p	35.6 ± 0.6	1.1 ± 0.1	11.4 ± 0.7	29.8 ± 0.5	18.2 ± 0.8	3.4 ± 0.1	0.6 ± 0.1
PS	35.7 ± 2.0	1.1 ± 0.1	11.9 ± 0.8	30.1 ± 1.0	17.6 ± 1.4	3.2 ± 0.1	0.5 ± 0.1

[a]PC, Phosphatidylcholine; other abbreviations as in Table 1.
[b]Data represent the mean ± SD of at least three determinations.

tion and possibly by hydrogen bonding. The addition of methanol will increase the dielectric constant and the repulsion of gradually dissociating negative charges, resulting in elution of lipids.

The analysis by gas–liquid chromatography of PS, obtained after the elimination of all protecting groups and purified by CM-Cellulose column chromatography, showed that PS and PC fatty acids compositions were the same (Table 2). This result seems to indicate that the coupling of particular molecular species is not preferred and that the double bonds were not affected under the mild oxidation conditions used.

PS was identified by infrared and ^1H NMR (Fig. 1), being the obtained spectra identical to those reported in the bibliography (13,28). PC and PS ^{31}P NMR spectra showed a sole signal at δ_P −1.244 and −0.265 ppm, respectively. The relative upfield shift of phosphate group of PC vs. PS can be correlated with the quaternary nitrogen of choline (29).

The methods previously described for obtaining PS by the phosphoramidite approach (20–23,25) have been applied to molecular species with the same fatty acid composition at the *sn*-1 and *sn*-2 positions of glycerol. This paper describes a new method, and with excellent yield, for the synthesis of PS with the same fatty acid composition at the *sn*-1 and *sn*-2 positions of glycerol as egg PC, using a phosphite–triester approach.

Phosphodiester (14) and phosphate–triester (14) methods have been described for the preparation of PS. Low yields, formation of undesirable products, and long reaction times have been some of the problems associated with these methodologies. The methods described here are faster, simpler, and more selective. It is based on the phosphite–triester method that has been proven to be more efficient in solid phase oligonucleotide synthesis (16,19). The separation of the unreacted materials also is easier due to the large differences on their polarity, and it could be performed using materials readily available. Moreover, phosphorothioate and other phosphate derivatives of PS can be prepared from the intermediate phosphite–triester.

On the other hand, PS also could be prepared in higher yields by the transphosphatidylation method using streptomyces phospholipase D (10). Nevertheless, the differences in

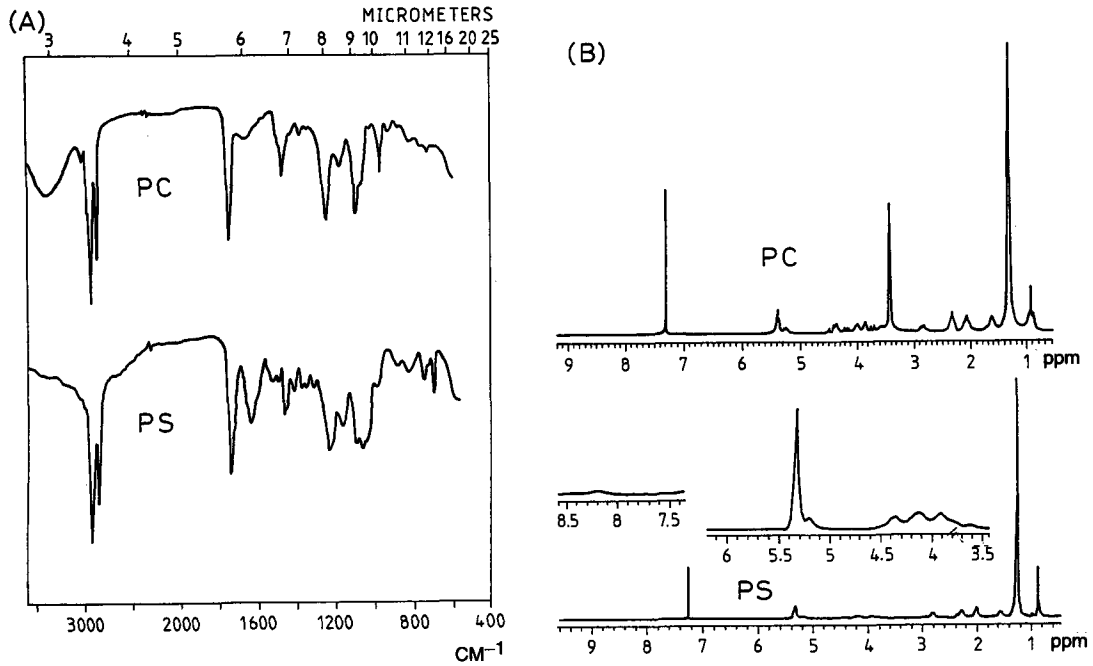


FIG. 1. (A) Infrared and (B) ^1H nuclear magnetic resonance spectra of phosphatidylcholine (PC) and phosphatidylserine (PS).

yields obtained with the method we propose and the transphosphatidylation viewed in the context of the costs of reagents indicate that the synthetic method described in this paper may be less expensive, depending upon the relative costs of phospholipase D from streptomyces species and additional labor needed for our method.

The protective groups would be easily cleaved in two steps. The cyanoethyl group was eliminated by addition of Et_3N/CH_3CN/pyridine mixture, and TFA was used to simultaneously eliminate the protecting groups of PS_p. It was not possible to use other functional protective groups which could be uniformly removed since phosphorous protecting groups cleavable in acid media are not available. A β-elimination mechanism for cyanoethyl group removal could yield phosphatidic acid as a by-product, but that secondary reaction did not take place since phosphatidic acid was not detected by TLC analysis.

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Effect of Medium-Chain Triglycerides on Calbindin-D9k Expression in the Intestine

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ABSTRACT: These studies determined the effect of the saturated fat source in infant formula on the expression of calbindin-D9k (CaBP-9k). Piglets were fed from birth to 8 d with milk or formula containing saturated fatty acids as medium-chain triglycerides (MCT), coconut oil, palm oil (Palm 1), or synthesized triglycerides with 16:0 directed to the *sn*-2 position (Palm 2). Levels of intestinal CaBP-9k mRNA were significantly ($P < 0.01$) higher in piglets fed formula with MCT than in piglets fed the other formula or milk; and higher in piglets fed the Palm-1 than in piglets fed Palm-2 formula. This is the first evidence that MCT alter piglet intestinal CaBP-9k mRNA.

Lipids 31, 547–549 (1996).

The effect of dietary fatty acids on small intestinal gene expression has received little attention. Enterocytes are the absorptive cells of the small intestine and are responsible for production of proteins involved in transepithelial transport and metabolism of nutrients (1). The enterocytes originate in the proliferative, crypt region of the villus, differentiate as they migrate toward the villus tip, and are then extruded into the gut lumen. Intestinal gene expression is known to follow a horizontal gradient of expression along the proximal to distal axis and variable expression along the crypt to villus axis (2,3).

Medium-chain triglycerides (MCT), containing 8:0 plus 10:0, are used as the source of saturated fatty acids in formula for preterm infants and other infants with reduced ability for fat absorption. Palmitic acid (16:0) is the major saturated fatty acid in human milk and is predominantly esterified at the center (*sn-2*) position of the milk triglycerides. Formula for term infants usually contains 12:0 plus 14:0 from coconut oils, or palm or other oils with 16:0 mainly esterified at the *sn*-1,3 positions of the triglycerides (4–6). The effect of MCT on the absorption of other nutrients is not known; however, previous studies have reported higher serum calcium concentrations in piglets fed formula with MCT than in piglets fed sow milk (7).

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Abbreviations: CaBP-9k, calbindin-D9k; MCT, medium-chain triglycerides; Palm-1 and Palm-2, 16:0 esterified predominantly to the triglyceride at *sn*-1,3 and *sn*-2, respectively.

Although little is known about the potential effects of dietary MCT on enterocyte metabolism, increased jejunal villus height and increased cell proliferation in the proximal small intestine (8,9), and higher alkaline phosphatase and lower sucrase-isomaltase activities (10,11) have been found in rats fed MCT than in rats fed long-chain triglycerides. MCT may influence nutrient absorption *via* nonspecific changes in intestinal morphology which encompasses alterations in enterocyte metabolism.

Calcium absorption is dependent on vitamin D and requires calbindin-D9k (CaBP-9k), a cytosolic calcium binding protein. CaBP-9k is involved in the intracellular movement of calcium (12,13). In response to low blood calcium, increased parathyroid hormone stimulates renal 1,25-dihydroxycholecalciferol production, resulting in increased intestinal CaBP-9k production and, consequently, increased intestinal calcium absorption (14). This report describes the results of studies determining if formula-feeding or the saturated fatty acid composition of formula, when compared to milk, influences piglet intestinal CaBP-9k expression.

METHODS

Animals and diets. Male Yorkshire piglets were randomly assigned to milk- or formula-feeding at birth ($n = 8$/group), and fed from birth to 8 d of age (15). The formula contained saturated fatty acids as 8:0 plus 10:0 (MCT), 12:0 plus 14:0 (coconut oil), or 16:0 esterified predominately to the triglyceride *sn*-1,3 (Palm-1) or *sn*-2 (Palm-2) position (Table 1) (7).

Tissue collection. The piglets were sacrificed after 8 d of feeding (7). The entire small intestine was dissected, measured, and divided into three equal segments. The upper third was flushed with ice-cold phosphate buffered saline (50 mmol/L phosphate, 100 mmol/L NaCl, pH 7.4) and immediately frozen in liquid nitrogen.

Extraction of RNA and Northern blot analysis. Total RNA was isolated from the upper third of the small intestine using guanidine thiocyanate followed by cesium chloride density gradient centrifugation (16). Total RNA (5 μg) was separated on 1% agarose denaturing gels containing 2.2 M formaldehyde and transferred to nylon membranes (Hybond-N; Amersham, Oakville, Ontario, Canada). The membranes were hybridized overnight in buffer containing 50% formamide, 5 ×

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TABLE 1
Saturated Fat Composition of the Experimental Diets[a] and the Effect on CaBP-9k Gene Expression in the Piglet Intestine

Fatty acids (% total)[a]	Diet				
	Sow milk	MCT	Coconut oil	Palm-1	Palm-2
8:0 + 10:0	0.2	31.1	6.3	1.3	1.0
12:0 + 14:0	3.8	0.8	25.3	6.3	4.1
16:0	30.5	4.1	6.3	26.8	29.6
18:0	4.4	2.6	2.9	4.6	3.1
18:1n-9	37.5	40.3	39.0	41.0	40.6
18:2n-6	11.1	16.0	15.6	16.4	16.4
18:3n-3	1.1	3.6	3.2	3.2	3.2
CaBP-9k[b] (Bq)	11.4 ± 5	19.7 ± 9.9[c]	10.3 ± 4.8	14.0 ± 7.7	5.2 ± 2.9[d]

[a]The formula had <0.13 mmol, and the sow milk had 0.5–1.0 mmol cholesterol/L. The sow milk, 16:0 predominantly esterified to the triglyceride *sn*-1,3 (Palm-1) or *sn*-2 (Palm-2) formula had 55, 4, and 70% 16:0 at the *sn*-2 position, respectively; MCT, medium-chain triglycerides.
[b]The relative abundance of calbindin-D9k (CaBP-9k) mRNA as determined by Northern blot hybridization. Values are in Bq, means ± SD, $n = 8$ per group.
[c]Significantly different from the sow milk, coconut oil, and Palm-2 groups ($P < 0.01$).
[d]Significantly different from the Palm-1 group ($P < 0.01$).

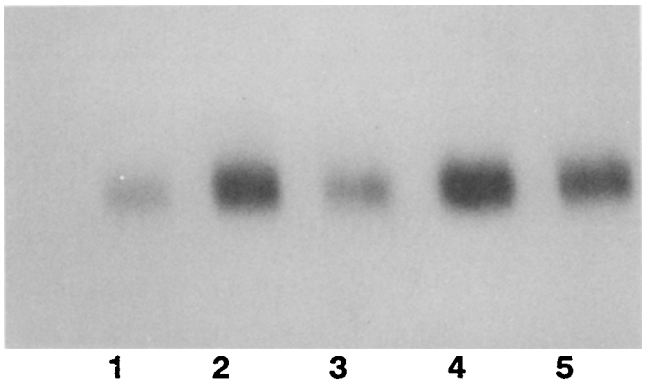


FIG. 1. Representative Northern blot of intestinal total RNA (5 μg) from 8-day-old piglets fed: Lane 1, 16:0 esterified predominantly to the triglyceride *sn*-2 formula; Lane 2, medium-chain triglyceride formula; Lane 3, coconut oil formula; Lane 4,16:0 esterified predominantly to the triglyceride *sn*-1,3 formula; and Lane 5, sow milk.

Denhardt's solution, 5 × sodium chloride, sodium phosphate, EDTA, 0.1% SDS, and 0.1 μg/L denatured salmon sperm DNA with a rat CaBP-9k cDNA (17) probe radiolabelled with [∝–^{32}P]dCTP (Amersham) by random priming (Lifetechnologies, Inc., Burlington, Ontario, Canada). The filters were washed, 42°C for 30 min in 1 × sodium chloride, sodium citrate and 0.1% SDS, then at 55°C for 30 min in 0.1 × sodium chloride, sodium citrate and 0.1% SDS, and then exposed to Kodak (Rochester, NY) XOMAT-AR film using an intensifying screen. The bands were assayed for radioactivity by Cerenkow counting. The levels of β-actin mRNA per μg RNA did not vary among the groups (data not included).

Statistical analysis. Results were compared among the groups of formula-fed piglets and between the formula-fed and sow-fed piglets using analysis of variance with tests of significant differences made using Number Cruncher Statistical System, version 5.01 (Number Cruncher, Kaysville, UT).

RESULTS

The relative intensities of the bands corresponding to the CaBP-9k mRNA abundance in piglets fed milk or formula are listed in Table 1. A representative Northern blot analysis from each diet group can be seen in Figure 1. Piglets fed the MCT formula had significantly ($P < 0.01$) higher CaBP-9k mRNA relative abundance than piglets fed sow milk, or those fed the coconut oil or Palm-2 formula. Piglets fed the Palm-2 formula had significantly lower CaBP-9k mRNA than piglets fed the Palm-1.

DISCUSSION

These studies are the first demonstration that dietary MCT have a significant effect on CaBP-9k mRNA abundance in developing intestine. It should be noted that these are preliminary studies and did not include measures of calcium absorption or changes in intestinal absorption. Although CaBP-9k may be regulated at transcriptional and post-transcriptional levels, mRNA abundance appears to correlate well with the amount of CaBP-9k protein produced (18). It is not known if the changes in CaBP-9k mRNA abundance among piglets fed the different diets were accompanied by changes in intestinal cell calcium translocation. The effect of dietary calcium on CaBP-9k expression is indirect, suggesting that differences in intraluminal calcium availability are unlikely to explain the difference in CaBP-9k mRNA. The changes in CaBP-9k mRNA, however, are consistent with the changes in serum calcium found in previous studies with these diets (7).

Dietary MCT are known to induce changes in intestinal morphology by increasing the rate of epithelial cell renewal, villus length, and crypt depth (8). Changes in intestinal enzyme activity induced by feeding MCT may also be a result of diet-induced changes in intestinal morphology (3,10). Diet-induced differences in epithelial cell turnover could be important if CaBP-9k mRNA abundance differs along the intestinal crypt to villus axis. In this regard, the expression of avian CaBP-28k in crypt compared to villus cells does vary with enterocyte differentiation and developmental stage (19). Whether the effects of dietary MCT on the piglet intestinal CaBP-9k mRNA abundance are specific for this gene or secondary to changes in intestinal morphology remains to be determined.

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ERRATUM

In *Lipids,* Vol. 31, no. 2 (February 1996), "Quantitative High-Performance Liquid Chromatography Analysis of Plant Phospholipids and Glycolipids Using Light-Scattering Detection," by G.A. Picchioni, A.E. Watada, and B.D. Whitaker, pp. 217–221, the sentence on page 220 "Also, ELS sensitivity and detection limits appear to vary ELSD among plant lipid classes" should read "Also, ELSD sensitivity and detection limits appear to vary widely among plant lipid classes."

ANNOUNCEMENTS

SPECIAL *LIPIDS* SUPPLEMENT—PROCEEDINGS OF THE ISSFAL 2ND INTERNATIONAL CONGRESS

Extra copies of the March *Lipids* Supplement—"Fatty Acids and Lipids from Cell Biology to Human Disease," the Proceedings of the 2nd International Congress of the ISSFAL (International Society for the Study of Fatty Acids and Lipids)—which includes 57 full manuscripts from participants in oral presentations at the Congress (332 total pages), are available for individual purchase at $60 each. Information: AOCS Press, P.O. Box 3489, Champaign, IL 61826-3489 (Phone: 1-217-359-2344, extension 128; Fax: 1-217-351-8091).

CALENDAR

June 1996

Symposium on Diet, Nutrition and Health: An Update, University of Western Ontario from June 16–19, 1996, sponsored by the Royal Society of Canada. Sessions on Diet and Cardiovascular Disease, Diet and Cancer, Diet and Selected Health Problems, Including Hypertension, Diabetes, Obesity, Kidney Disease, Osteoporosis and Alcohol Abuse, as well as Diet and Health Maintenance in People at Various Stages of Life, Including the Elderly. Information: The Centre for Human Nutrition, The University of Western Ontario, London, ON N6A 5C1, Canada (Phone: 519-661-3096; Fax: 519-661-4006).

July 1996

European Thrombosis Research Organisation and the International Society for the Study of Fatty Acids and Lipids in cooperation with Summer University of Maastricht, July 10–12, 1996, University of Maastricht, The Netherlands, presents "Lipids, Membranes and Thrombosis (fundamental basis of cardiovascular disease and its prevention)." Information: Gerard Hornstra, Department of Human Biology, P.O. Box 616, 6200MD Maastricht, The Netherlands (Phone: +31-43-88 16 25; Fax: +31-43-67 09 76; E-mail: G. Hornstra @ HB.RuLimburg.NL).

September 1996

37th International Conference on the Biochemistry of Lipids, Antwerp, Belgium, Sept. 4–7, 1996. Themes: enzymes and transfer proteins related to lipoprotein metabolism, aspects of protein lipidation, and peroxisomal lipid metabolism. Poster communications may relate to the three conference themes or any other area of lipid metabolism. Information: Dr. P. van Dyck, Secretariat 37th ICBL, Department of Biochemistry, Physiology and Genetics, Division of Human Biochemistry, State University Centre Antwerp, Groenenborgerlaan 171, B2020 Antwerp, Belgium (Phone: 32 3 218 0329; Fax: 32 3 218 0326).

7th International Congress on Phospholipids, Sept. 9–11, 1996, Brussels, Belgium. Program topic areas include Growth, Death and Cancer; Choline, Choline Phospholipids and the Brain; Importance of Phospholipids in Health and Disease. Information: AOCS Meetings Department, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

IV. International Dermatology Symposion Berlin, Sebaceous Gland and Its Disorders, Basic and Clinical Research, Clinical Entities and Treatment, Sept. 13–15, 1996, University Medical Center Benjamin Franklin. Information: Docent Dr. Ch. C. Zouboulis, Department of Dermatology, University Medical Center Benjamin Franklin, The Free University of Berlin, Hindenburgdamm 20, D-12200 Berlin, Germany (Phone: 49-30-8445-2808; Fax: 49-30-8445-4262).

Training in Free Radical Methodologies, Sept. 23–27, 1996, CERLIB, University of Grenoble, Grenoble, France. Production, damages, repair: ESR, nitric oxide, DNA oxidation, DNA repair, apoptosis, chemical-trapping, lipoprotein oxidation, protein oxidation, cellular production and toxicity. Information: Arlette Alcaraz, Laboratoire de Biochimie C, CHU Grenoble BP 217, 38043 Grenoble Cédex 9, France (Phone: 33 76 76 57 54; Fax: 33 76 76 56 64; E-mail: CERLIB@ujf-grenoble.fr).

October 1996

Frontiers in Lipid and Lipoprotein Research: Basic Science, Analytical, Clinical, and Public Health Applications, Oct. 4–6, 1996, Stouffer Renaissance Hotel, Dallas, Texas. Sponsored by the Lipids and Lipoproteins Division of the

American Association for Clinical Chemistry, the Centers for Disease Control and Prevention, and the American Heart Association. An integrated review of relevant recent advances in basic science, measurement technology, epidemiological and clinical research, and public health issues will be presented. This review will help define state-of-the-art techniques for understanding and preventing morbidity and mortality due to heart disease. Information: Paula M. Steiner, Medical Research Laboratories, 2 Tesseneer Dr., Highland Heights, KY 41076 (Phone: 606-781-8877, Extension 252; Fax: 606-781-9310; E-mail: lipf@cehehll.em.cdc.gov).

American College of Nutrition, 37th Annual Meeting, Oct. 11–13, 1996, San Francisco, California (Phone: 212-777-1037; Fax: 212-777-1103).

Certification Board for Nutrition Specialists, Oct. 11, 1996, San Francisco, California. Certifying examination for nutritionists and dieticians with advanced degrees. Information: CBNS, 1562 First Avenue, Box #258, New York, NY 10028 (Phone: 212-332-0774 (leave message); Fax: 212-777-1103).

Seminar: Interconversion of Polyunsaturated Fatty Acids, and Their Functions in Membranes, Oslo, Norway, Oct. 11–12, 1996. Information: Georg Lambertsen, Secretary, Lipidforum, Breistølen 1, N-5035, Bergen, Norway (Phone: 4755 + 32 89 56; Fax: 47 55 + 32 57 24).

November 1996

AOCS Conference on PUFA in Infant Nutrition: Consensus and Controversies, Nov. 7–9, 1996, Barcelona, Spain. Information: Susan E. Carlson (E-mail: scarlson@utmem1.utmem.edu); Robert A. Gibson (E-mail: rgibson@paed_nnu.fmc.flinders.edu.au); Berthold Koletzko (Fax: 49-89-5160-4733); or AOCS Meetings Department, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

March 1997

CERLIB 3rd Winter Research Conferences, Les Arcs 1800, France, March 9–14, 1997. Proteins and Free Radicals, from Radical Enzymes to Damages; Oxidative Effects of Radiations on Biomolecules and Cells; Oxidative Stress and Apoptosis. Information: Arlette Alcaraz, Laboratoire de Biochimie C, CHU Grenoble BP 217, 38043 Grenoble Cédex 9, France (Phone: 33 76 76 57 54; Fax: 33 76 76 56 64; E-mail: CERLIB@ujf-grenoble.fr).

June 1997

The 19th Nordic Lipid Symposium, Ronneby, Sweden, June 15–18, 1997. Information: Georg Lambertsen, Secretary, Lipidforum, Breistølen 1, N-5035, Bergen, Norway (Phone: 47 55 + 32 89 56; Fax: 47 55 + 32 57 24).

July–August 1997

Fourth International Congress on Essential Fatty Acids and Eicosanoids, July 27–August 1, 1997, Edinburgh, Scotland. Information: Secretariat, c/o Meeting Makers, 50 George St., Glasgow G1 1QE, Scotland, United Kingdom.

REVIEW

Uses of Biotechnology in Modifying Plant Lipids

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ABSTRACT: This review discusses fatty acid modification of oilseeds with additional emphasis on production of oxygenated derivatives. In a relatively short period, less than a decade, our understanding of the enzymes involved in plant fatty acid synthesis has increased to the point where we understand how they might be used in oilseed modification. Further, through modern molecular biological techniques, the actual genes for many of these important enzymes have been cloned. Use of genetic transformation systems has allowed us to fundamentally alter the normal biosynthetic pathways in highly specific ways, in manners that would be either difficult or impossible using traditional breeding techniques. Alteration of plant lipid biosynthesis is not restricted to using genes from the plants themselves, but interspecies transfer is possible, either from completely unrelated plant species (often of no commercial value but possessing unusual biochemical properties) or from animals, fungi, and prokaryotic organisms. In this way "designer" plants possessing altered metabolism, tailored to the interests or needs of certain industries, nutritionists, and the consumer can be created.
Lipids 31, 557–569 (1996).

In recent years, the field of plant molecular biology has advanced rapidly, moving from an area of academic research to applied use in agro-industries. Exciting new prospects in agricultural production are being developed through the use of plant metabolic engineering. The myriad of plant secondary metabolites and primary metabolites, estimated to number in excess of 20,000 unique chemical structures (1), potentially could be cultivated and harvested in commercial crop species through the transfer of critical genes within a metabolic path. Presently developed crops, maximized for agronomic productivity, could be turned into chemical factories *via* a redirection of metabolic flow, utilizing the sun's radiant energy toward the production of desired high-value metabolites. Recent success in the engineering of select oilseed crops for an increase in the production of desired fatty acids (FA) provides evidence of the commercial potential to be exploited through the metabolic engineering of plants.

The cloning of plant and animal genes and the sequencing of their DNA have now become commonplace, and the list of cloned and identified genes has grown impressively and continues to do so (see Table 1 for a list of genes encoding plant lipid biosynthetic genes now cloned). In addition to the genes listed in Table 1, there is a whole array of other genes now known from animals, fungi, and prokaryotes which for reasons of brevity are not listed here. Large databases of known DNA sequences exist and are available for use by research scientists, i.e., through the NCBI GenBank in the United States, the DNA Data Bank of Japan (DDBJ), and the European Molecular Biology Laboratory (EMBL) database. The increase in information deposited in the databases is quite dramatic. For example, in the months between June and October 1995, the number of bases deposited in Genbank increased by 21% to 3.85×10^8 bases total and the total number of individual sequences by 31% to 556,000 clones. As little as five years ago, none of the FA biosynthetic enzymes had been fully characterized and none of the genes had been cloned. Table 1 shows the current standing in cloned and fully characterized plant FA biosynthesis genes and, as can be seen, many of the genes necessary for altering lipid biosynthesis are now available.

The high level of interest in the area of biotechnological alteration of plant lipids for science and industry has become apparent from the publication of several excellent reviews (1–6). Several approaches are presently being pursued both by industry and academia to alter lipid metabolism in transgenic plants. Changing the FA composition of seed storage, triacylglycerols (TAG) can increase the economic value of the plant-derived oil. The value of oil crops may be enhanced simply by removal of undesirable characteristics from the oil, an example being the reduction of high levels of saturated FA in food oils. Alternatively, the plant oil also may be modified for the accumulation of novel and unique high value substances for use in the pharmaceutical, chemical, or other industries.

The predominant FA contained in the TAG of most oilseed crops are palmitic (16:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) FA. A small percentage of stearate (18:0) is also present (7). As exemplified through the work of Knutzon and colleagues (8), FA metabolic modification can be accomplished by altering the levels of proteins expressed by na-

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Abbreviations: ACCase, acetyl-CoA carboxylase; ACP, acyl carrier protein; DAG, diacylglycerol; DAGAT, diacylglycerol acyltransferase; FA, fatty acid; FAE, fatty acid elongase; FAS, fatty acid synthase; G3PAT, glycerol-3-phosphate acyltransferase; HEAR, high erucic acid rapeseed; KAS, β-ketoacyl synthase; LOX, lipoxygenase; LPAAT, lysophosphatidic acid acyltransferase; LPC, lysophophatidylcholine; LPCAT, acyl-CoA lysophosphatidylcholine acyltransferase; PA, phosphatidic acid; PC, phosphatidylcholine; TAG, triacylglycerol; VLCFA, very long chain fatty acids.

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TABLE 1
A Brief Summary of Some of the Currently Cloned Plant Lipid Biosynthesis and Modification Enzymes Available as of Mid-1995[a]

Enzyme	Organism	Number of clones
Acetyl-CoA carboxylase	*Arabidopsis thaliana*	6
	Brassica napus	2
	Triticum aestivum	2
β-Keto acyl synthase	*Hordeum vulgare*	2
acyl carrier proteins	*A. thaliana*	3
	B. napus	12
	B. campestris	1
	Cuphea lanceolata	3
	Hordeum vulgare	6
	Linum usitatissimum	1
	Ricinus communis	1
	Spinacia oleracea	1
	Zea mays	1
Stearoyl ACP-desaturase	*B. napus*	1
	L. usitatissimum	1
	R. communis	1
Acyl-ACP thioesterase	*A. thaliana*	2
	B. napus	1
	C. lanceolata	1
Fatty acid desaturases		
FAD2	*A. thaliana*	3
	Glycine max	2
FAD3	*A. thaliana*	3
	G. max	2
	Limnanthes douglasii	1
	Nicotiana tabacum	1
FAD6	*A. thaliana*	1
	S. oleracea	1
Lipoxygenase	*A. thaliana*	2
	Cucumis sativus	1
	G. max	15
	H. vulgare	3
	Lens culinaris	1
	Lycopersicon esculentum	2
	Phaseolus vulgaris	2
	Pisum sativum	6
	Solanum tuberosum	1
	Z. mays	2
P450 Hydroxylase	*Z. mays*	2
Hydroxylase	*R. communis*	1
Allene oxide synthase	*L. usitatissimum*	1

[a]The number of gene clones for some of these enzymes might be somewhat inflated in some cases due to different labs cloning and sequencing essentially the same cDNA or genomic DNA clones from different libraries, different plant genotypes, etc. Detailed information on all of these and for other lipid biosynthesis enzymes from animals, fungi, and prokaryotes is available from any of the Internet WWW (worldwide web) databases mentioned in the introduction. Information on NCBI GenBank is available on the WWW (at http://www.ncbi.nlm.nih.gov/). ACP, acyl carrier protein.

tive genes within a particular plant species. Alternatively, as exemplified by a rapeseed cultivar engineered for high lauric acid accumulation (9,10), new and valuable FA metabolites, normally not produced by a particular commercial oilseed crop, can be produced by the transfer of genes from one species to another. The genes used to metabolically engineer oilseed crops need not be limited to the plant kingdom, for there are examples of plants engineered for altered FA metabolism *via* the introduction of animal and yeast genes (11,12). In addition, Δ6, Δ9, and Δ12 desaturases have been cloned from the cyanobacterium *Synechocystis* (13,14), which may prove useful in the genetic engineering of oilseeds for altered FA composition.

With many genes involved in plant lipid biosynthesis isolated and characterized, there is, within the next five years, likely to be a dramatic increase in the number of engineered oilseed crops in commercial production. In this review we will focus on the alteration of plant lipids through the use of biotechnological techniques, discussing the current status and future prospects for this field of applied technology.

PLANT FA METABOLISM

Figures 1 and 2 provide a summary of the current understanding in plant FA and TAG biosynthesis, showing the major biochemical steps and compartmentalization of the process.

First steps in de novo *FA synthesis.* Most *de novo* plant FA synthesis occurs in the plastids and begins with the first committed step, a reaction between acetyl-CoA and a bicarbonate ion. The reaction, catalyzed by acetyl-CoA carboxylase (ACCase, E.C. 6.4.1.2,), results in the formation of malonyl-CoA and is believed to be rate-limiting for the production of FA (15). Table 1 shows that several ACCase genes have been cloned from several plant species. The genes should aid the elucidation of ACCase's regulatory role in plant FA metabolism.

The next stage of FA synthesis, the initiation of an FA carbon chain, is catalyzed by 3-ketoacyl-ACP (ACP = acyl carrier protein) synthase (β-ketoacyl synthase) (KAS) III associated within a Type II dissociable FA synthase (FAS) II complex (16). In this reaction, malonyl-ACP condenses with an acetyl-CoA molecule. The initial product of the condensation, a β-ketoacyl-ACP molecule, is converted to butyryl-ACP (butyric acid esterified to ACP) *via* a reduction, dehydration, and second reduction of the β-ketoacyl derivative. The reactions following the condensation are catalyzed by the enzymes associated in the FAS II complex. Table 1 shows that KAS genes and ACP have been isolated from several plant species.

FA chain elongation. Elongation of the FA chain, from 4-C butyryl-ACP to 16-C palmitoyl-ACP, proceeds *via* two carbon additions with malonyl-CoA supplying the carbons. Each malonyl-ACP (malonyl-CoA is transferred to an ACP molecule prior to condensation) condensation with the growing fatty acyl-ACP, up to 16-C palmitoyl-ACP, is catalyzed by KAS I, associated within the FAS II complex (17). The conversion of the 16 carbon FA thioester, palmitoyl-ACP, to the 18 carbon FA thioester, stearoyl-ACP, is catalyzed by KAS II associated in the FAS II complex (18). Stearoyl-ACP is largely converted to the monounsaturated FA thioester oleoyl-ACP by the activity of stearoyl-ACP desaturase (E.C. 3.1.2.14) (19), a soluble enzyme located in the chloroplast stroma which introduces a double bond between the ninth and tenth carbons of the FA chain.

The mechanism by which plants control the FA chain length involves a complex kinetic interplay between enzymes competing for FA substrates. As an FA chain is being built

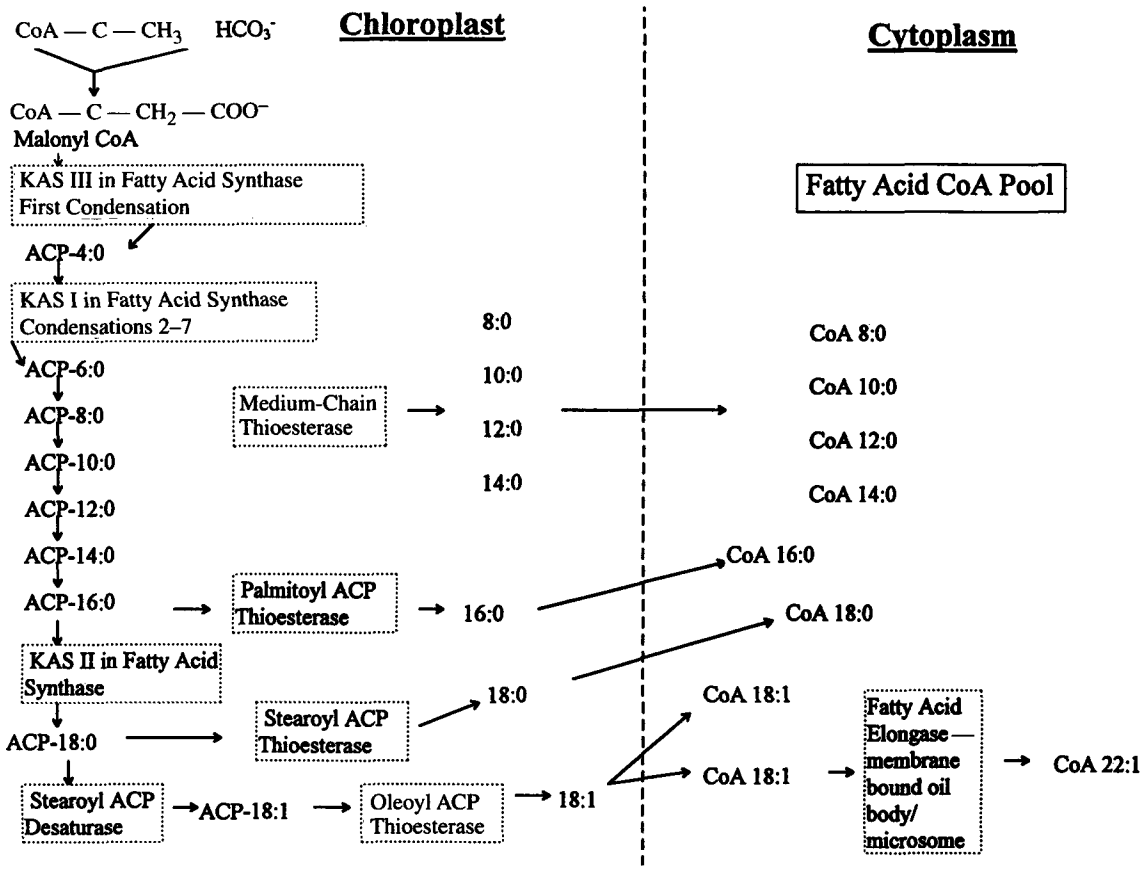


FIG. 1. Schematic overview of fatty acid synthesis in plant tissues. See text for detailed description. KAS, β-ketoacyl synthase; ACP, acyl carrier protein.

(C_4 to C_{18}), it is attached to an ACP *via* a thioester linkage. Severing of the thioester linkage can be brought about at specific substrate chain lengths by the activity of either an acyl-ACP hydrolase (thioesterase) or an acyltransferase. The result of either enzymatic activity is the termination of FA chain length extension. The "competition" for substrate is thus a competition between the termination of synthesis, a function of thioesterase and transferase activity, and extension, a function of the KAS I and II isoforms. An example of the complex kinetic interplay related to the FA chain length can be viewed with the California bay *(Umbellularia californica)*. California bay's TAG profile in seeds may consist of up to 70% lauric acid (12:0). These short-chain FA are produced by a thioesterase which shows maximal hydrolytic activity on 12C-ACP thioesters (9,20). The medium-chain thioesterase thus diverts a portion of FA from the elongation pathway and forms lauric acid.

FA of 18 and 16 carbons in length tend to predominate in the tissues of most commercially grown plant species. C_{18} FA generally range from 60 to 95% and C_{16} FA range from 5 to 45% of total FA of such tissues (21), depending on plant species. However, unique FA of unusual chain length (i.e., $< C_{16}$ or $>$ than C_{18}) or with atypical side groups (hydroxyl, epoxide, keto, etc.) can often be found in noncommercial members of the plant kingdom, although usually these lipids are maintained in the storage TAG (22).

Extra-plastidial metabolism of FA. FA molecules, formed in the chloroplast stroma, are released and cross the membrane by an unknown mechanism. As the FA cross the membrane, they are converted to fatty acyl-CoA esters through the activity of an acyl-CoA synthetase, located on the outer chloroplastic membrane (3). In the cytoplasm, the fatty acyl-CoA form a pool from which they have several possible fates.

In some cruciferous oilseed crops, 18:1-CoA from the acyl-CoA pool may be elongated to eicosenoyl-CoA (20:1-CoA) or erucoyl-CoA (22:1-CoA), known as very long chain FA (VLCFA). The reactions are thought to be catalyzed by a multiprotein, microsomal, membrane-bound complex (23). Recently the cDNA sequence of a constituent FA elongase (FAE) I of the multiprotein elongation complex has been isolated from *Arabidopsis* (24). The protein sequence deduced from the cDNA shows sequence homology with chalcone synthase, stillbene synthase, KAS III from *Escherichia coli*, and KAS III from spinach.

The mechanism of VLCFA synthesis involves the condensation of 18:1-CoA with malonyl-ACP, forming the β-ketoacyl derivative. As previously described, the steps following the initial condensation involve the reduction and dehydration of the β-ketoacyl molecule, followed by a second reduction. The resulting 20:1 fatty acyl-CoA molecule then may be acted upon *via* the same mechanism of the elongation complex producing 22:1-CoA. The mechanism of the FAE I con-

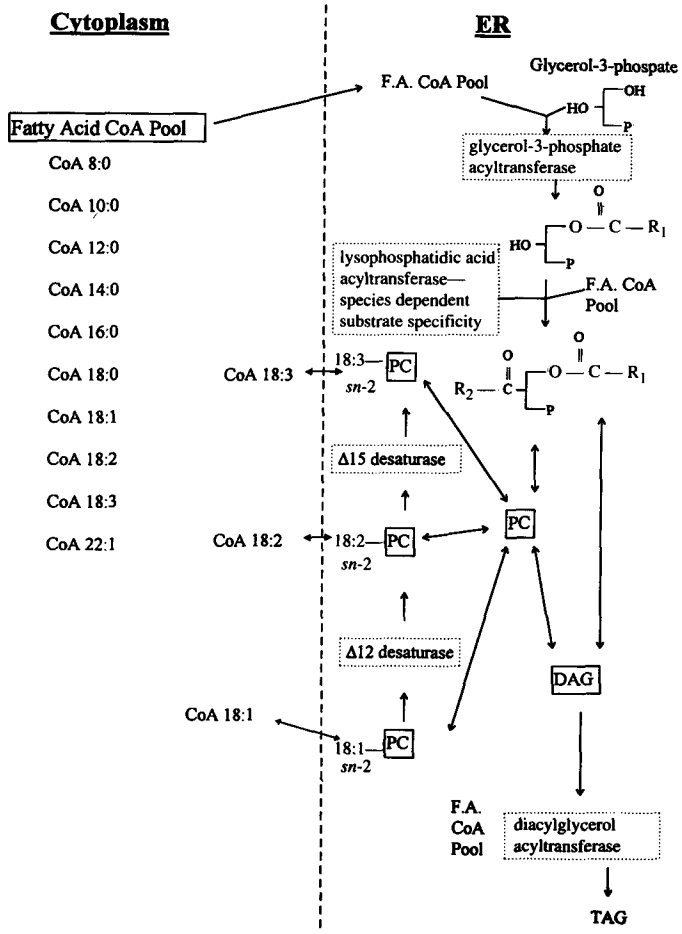


FIG. 2. Schematic overview of phospholipid and triacylglycerol (TAG) synthesis in plants. See text for detailed description. F.A., fatty acid; PC, phosphatidylcholine; DAG, diacylglycerol.

densation resembles the reaction catalyzed by KAS III, where malonyl-ACP condenses with an acyl-CoA (acetyl-CoA). The reaction differs from the 4-carbon to 18-carbon elongation where the malonyl-ACP is condensing with fatty acyl-ACP molecules (24). In select cruciferous oilseed crops, VLCFA are present in the fatty acyl-CoA pool.

Phospholipid and triglyceride synthesis. The fatty acyl pool serves as a source of FA used in the production of phospholipids and TAG. The phospholipids, phosphatidic acid (PA) and phosphatidylcholine (PC), are precursors to diacylglycerol (DAG) which, in turn, is the immediate precursor of TAG. PC, the major substrate of the oleoyl desaturase and the linoleoyl desaturase, is also a source of polyunsaturated FA for the fatty acyl-CoA pool. Formation of TAG begins with the acylation of glycerol-3-phosphate at the *sn*-1 position. The enzyme catalyzing the reaction, glycerol-3-phosphate acyltransferase (G3PAT; E.C. 2.3.1.15), is located in the microsomes and is most active in acylating saturated FA (25). The resulting molecule, lysophosphatidic acid, is then acted upon by a second microsomal enzyme, the lysophosphatidic acid acyltransferase (LPAAT; E.C. 2.3.1.51). The reaction forms PA through the transfer of a fatty acyl group to the *sn*-2 position. LPAAT is usually highly selective, showing highest enzymatic activity for the acylation of unsaturated FA (26–28). PA may be converted to PC by the addition of a choline headgroup or it may be dephosphorylated, forming DAG, by the activity of PA phosphatase (E.C. 3.1.3.4). DAG acyltransferase (DAGAT; E.C. 2.3.1.20), located in the microsomal membranes, converts DAG to TAG by acylating the *sn*-3 position with an acyl-CoA substrate from the fatty acyl-CoA pool.

FA desaturation. PC molecules are acted upon in the microsomes by the membrane-bound desaturases, oleoyl desaturase and linoleoyl desaturase. These desaturases convert the fatty acyl group at the *sn*-2 position from oleic to linoleic acid and from linoleic to linolenic acid, respectively. A reversible acyl exchange reaction, involving the acyl-CoA dependent fatty acylation of lysophosphatidylcholine (LPC) at the *sn*-2 position or, conversely, the transfer of an acyl group from the *sn*-2 position of PC to CoA, is catalyzed by acyl-CoA-LPC acyltransferase (LPCAT). The polyunsaturated thioesters (18:2 and 18:3), produced at the *sn*-2 position, are thus made available to the fatty acyl-CoA pool *via* the activity of the LPCAT (29–32). PC also may be converted to DAG, and subsequently to TAG, in a reversible reaction, through the activity of choline phosphotransferase (33,34).

PLANT GENETIC ENGINEERING

Altering a plant's biochemistry by genetic engineering requires that genes of interest be delivered into plant cells, stably integrated into the genome, and readily expressed by the cell. Transformed plant cells must then be converted to healthy, fertile adult plants. A variety of methods have been developed to accomplish the task of plant genetic engineering. Two of the most developed and successful methods involve the use of *Agrobacterium* (35) and particle bombardment (gene gun) (36).

A gene construct, prepared for introduction into a plant's genome, is equipped with a promoter region and a poly-A addition site. This enables the gene to be transcribed and translated normally in the cell. Use of tissue-specific gene promoters can allow for the expression of genes in desired tissues or at specific developmental stages. The capability for targeted gene expression is of immense importance, particularly with regard to altering FA metabolism, as the introduction of a gene can radically alter a plant's normal biochemistry. For commercial use of genetic engineering in common crop plants, it is desirable to target the alteration of lipid metabolism only to the seed, so as not to effect desirable agronomic qualities of the rest of the plant. The use of seed-specific promoters in gene constructs (37), introduced into plant cells for the purpose of altering the FA profile of seed TAG, ensures that the spatial expression of the gene occurs only in seeds. In addition, the seed-specific promoters allow for the temporal expression of the gene to coincide with the time at which the seed's metabolism is highly active in TAG production.

The orientation of a gene, with respect to the promoter, may be varied when inserted into the plant genome. A gene

oriented with the N-terminal translating region immediately succeeding the promoter is said to be of the "sense orientation" while the reverse orientation, with the C-terminal translating region immediately succeeding the promoter, is called "anti-sense." The "sense orientation" corresponds to the native orientation of a gene, and the "anti-sense orientation" corresponds to the reversal of a gene's native orientation.

When inserted into a plant genome, the sense gene construct normally produces the enzymatic activity encoded by the gene. The biochemical activity can be new to the plant, if the gene is not native or, alternatively, if the gene is native to the plant, the introduction of additional gene copies may raise the level of the native enzyme beyond standard levels. This has the potential of increasing substrate to product conversion at a particular biochemical step (sense upregulation). If a gene is inserted into the genome in the "anti-sense orientation," the effect may be to reduce the levels of enzymes that are highly homologous to the introduced gene within (38,39). A reduction in enzyme levels may cause a reduction of substrate flow through the biochemical step catalyzed by the affected enzyme (anti-sense downregulation). At times the use of the "sense orientation" has had the unexpected effect of decreasing the level of homologous enzymes. This phenomenon is termed "co-suppression" or "trans-switching" (40). Though the mechanism of co-suppression is not understood, it has become another method used in downregulation of specific genes. Any of the genetic engineering phenomena just mentioned (anti-sense downregulation, co-supression, or sense upregulation) may serve as a tool for affecting the accumulation of desired FA.

ALTERATION OF SATURATED/UNSATURATED FA LEVELS

A variety of molecular strategies may be employed to alter the saturated and unsaturated FA levels in oilseed crops. One strategy for increasing stearic acid levels in oilseed crops could employ the use of anti-sense supression or co-supression to reduce or knock out the activity of stearoyl-ACP desaturase (see Table 1 for cloned ACP). Stearoyl-ACP desaturase is the enzyme responsible for converting stearoyl-ACP (saturated FA) to oleoyl-ACP (unsaturated FA). Another possible metabolic target with the potential for increasing stearic acid accumulation is the stearoyl-ACP thioesterase (see Table 1). The enzyme's substrate to product conversion (converting stearoyl-ACP to free stearate) could be increased (upregulated) by sense-orientated reintroduction of the stearoyl-ACP thioesterase. An increase in stearate release from the ACP before stearoyl-ACP desaturase can act, effectively bypasses the conversion of saturated to unsaturated FA. A combination of the two strategies would likely lead to an even greater accumulation of stearic acid.

The first successful genetically engineered alteration of FA metabolism involved the modification of saturated/unsaturated FA levels in rapeseed. The levels of a saturated FA, stearic acid, was increased at the expense of a monounsaturated FA, oleic acid (8). Knutzon and co-workers (8) used the "anti-sense" supression strategy to reduce levels of the stearoyl-ACP desaturase in rape. The decrease in stearoyl-ACP desaturase levels resulted in a decrease in the conversion of stearoyl-ACP to oleoyl-ACP. This created a metabolic "traffic jam" at the biochemical step where saturated FA is converted to unsaturated FA and, in turn, allowed more stearate to be released from the ACP *via* the activity of stearoyl-ACP thioesterase. The anti-sense supression strategy ultimately resulted in an engineered rapeseed line that accumulated up to 40% stearic acid (normally not an abundant constituent of rapeseed oil) in its seed TAG. Stearic acid has the potential to be used in confectionary foods (as a cocoa butter substitute) and in the pharmaceutical and cosmetic industries (3). Though rapeseed has proven to be the easiest oilseed crop to genetically alter and regenerate, there is the potential for replicating the mentioned metabolic engineering strategies in other oilseeds (soybean, sunflower, peanut), especially as a wide range of these genes now have been cloned. Table 2 shows some of the currently modified oilseed crops.

Plant FA metabolism also may be engineered to alter the levels of unsaturated FA. The target enzymes for this goal include the stearoyl-ACP desaturase, oleoyl desaturase, and linoleoyl desaturase. Increasing the oleic acid content of seed oil at the expense of linoleic and linolenic acids has been set as a major goal of many researchers. A reduction in the levels

TABLE 2
A Brief List of Some of the Agronomically Important Oilseed Crops That Have Been Genetically Modified and Their Lipid Biosynthesis Pathways Altered

Crop species	Introduced gene	Genetic alteration	Reference
Brassica napus	Stearoyl ACP[a] desaturase in anti-sense orientation	Stearic acid accumulation	7
	C_{12} Acyl-[ACP] thioesterase	Lauric acid accumulation	4, 8
	C_{10} Acyl-[ACP] thioesterase	Capric acid accumulation	121
	ω3 and ω6 Fatty acid desaturase in anti-sense orientation	Lowered 18:2 and 18:3	4, 9, 41
Glycine max	ω3 and ω6 Fatty acid desaturase in anti-sense orientation	Lowered 18:2 and18:3	3, 41

[a]ACP, acyl carrier protein.

of the di- and trienoic polyunsaturates would increase the oxidative stability of an oil along with reducing processing (hydrogenation) costs (41). In the past few years, both the microsomal and plastidial forms of a variety of desaturases have been cloned from a range of plants (Table 1). The increase in oleic acid content could be accomplished *via* the reduction of the oleate desaturase (FAD2) levels using anti-sense supression or co-supression. Researchers at DuPont have demonstrated the validity of this strategy in both rapeseed and soybean (41). In rapeseed lines engineered with anti-sense cDNA of the oleate desaturase, levels of seed oil oleate were reported to be 83% (compared with 63% in control). The engineered rapeseed was crossed with a mutant line containing 77.8% oleate in seed oil, and the resultant cross had an oleate seed oil content of 88%. In soybean, anti-sense engineering produced a line of soybean with 78.9% oleate in seed oil (compared with 22% in control) (1).

Raising the levels of linolenic acid in seed oil serves as another potential goal. If produced in economic quantities, linolenic acid could compete with petrochemicals for use in paints, varnishes, coatings, and drying oils (1). In soybean seed oil, 18:3 and 18:2 levels usually run around 10 and 60%, respectively. A strategy for increasing linolenic acid in TAG employs increasing the level of linoleoyl desaturase (FAD3). Sense-oriented reintroduction of extra copies of the linoleoyl desaturase gene should increase enzyme levels and increase the conversion of 18:2 to 18:3. The increase in linolenic acid levels in the fatty acyl pool should, in turn, increase the percentage of linolenic acid in TAG.

Possible strategies for maximizing the linolenic acid levels involve a combined strategy of up- and downregulation of critical genes. This would involve multiple gene insertions. Reducing levels of the two thioesterases involved in freeing saturated FA (palmitoyl-ACP thioesterase and stearoyl-ACP thioesterase) should provide FA, normally destined to be incorporated into TAG as saturated FA, to be available for unsaturation. Anti-sense supression or co-supression of each gene should accomplish the goal. The reduction of the thioesterase levels combined with an increase in levels of the three enzymes involved in desaturation (stearoyl-ACP desaturase, oleolate desaturase, and linoleate desaturase) could maximize linolenic acid levels.

UNUSUAL FA PRODUCTION

There are a number of unusual FA that accumulate in the seed oil of various plants which might be useful if the genes encoding the key biosynthetic enzymes were transferred into oilseeds. We will cite one example here and focus particularly on oxygenated FA and derivative synthesis later in the review. One unusual FA, petroselinic acid (*cis*-6-octadecenoic acid), an isomer of oleic acid, has its double bond between the sixth and seventh carbon. The difference in double-bond positioning on the carbon chain alters the melting point, which is 33°C for petroselinic as opposed to 12°C for oleic acid. The higher melting point means that petroselinic acid is solid at room temperature and makes it potentially useful as a component of margarines and shortenings. The industrial potential of petroselinic acid as a chemical feedstock exists with the products produced by its ozone-catalyzed oxidation and scission products—lauric acid (dodecanoic acid, 12:0) and adipic acid (hexanedioic acid, 6:0). Lauric acid is used in detergents, and adipic acid is used in the production of nylon 6,6 (1,3).

Petroselinic acid, not produced in any commercial oilseed crop, may make up as much as 85% of the total FA in the Umbelliferae, Araliaceae, and Garryaceae plant families. Thus, knowledge of the biochemical steps involved in petroselinic acid production and isolation of the genes responsible for those steps are precursors to engineering oilseed crops for its production. Information gleaned regarding petroselinic acid production indicates that three genes may be involved (42). A palmitoyl-ACP desaturase, which has been cloned (43), introduces the double bond between the fourth and fifth carbons of palmitic acid. This is followed by activity of a postulated condensing enzyme, specific for the $cis^4$16:1 molecule, that elongates the substrate to $cis^6$18:1 petroselinic acid. Finally, release of the petroselinic acid molecule from the ACP molecule may necessitate a unique thioesterase. Thus, engineering of an oilseed crop for significant petroselinic acid accumulation would apparently require the introduction of at least three genes, not all of which have yet been cloned.

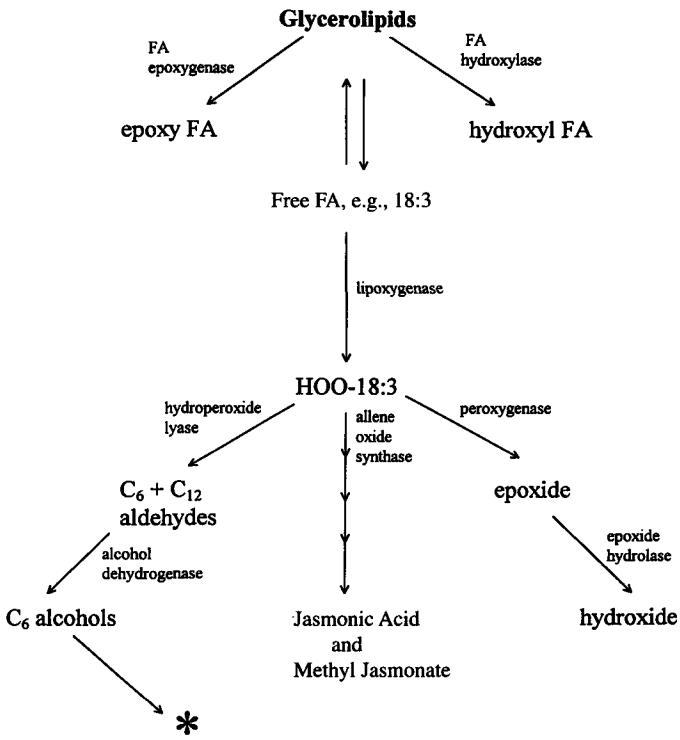
ALTERATION OF FA CHAIN LENGTH

Another thrust in the engineering of oilseed metabolism involves altering the chain lengths of FA that normally accumulate in seed oil. As stated previously, researchers at Calgene have successfully engineered oilseed rape to produce up to 40% lauric acid with no apparent effect on agronomic qualities (9). Production of medium-chain FA was brought about with the introduction of a medium-chain thioesterase (sense-orientation) under control of a seed-specific promoter, introducing the new enzymatic activity into rapeseed. The thioesterase, which shows maximal activity on 12:0-ACP, hydrolyzes the thioester linkage connecting the 12 carbon FA and ACP. The FA, once separated from the ACP, is no longer a substrate for elongation. It joins the fatty acyl-CoA pool in the cytoplasm and is eventually incorporated into TAG (9,20). Due to the absence of any activity of a native rapeseed LPAAT for lauric acid introduction at the *sn*-2 position of glycerol, the maximal lauric acid production was limited. In order to boost the production of lauric acid in rapeseed, it was hypothesized that an LPAAT (specific for medium-chain, saturated FA) if introduced into oilseed rape, would boost the accumulation of lauric acid in TAG. Researchers at Calgene have recently purified the LPAAT protein from immature coconut endosperm (44,45), which subsequently led to the cloning of the corresponding gene. Engineering of the lauric acid-producing rapeseed lines with the LPAAT gene has significantly increased the incorporation of lauric acid at the *sn*-2 position of TAG (H.M. Davies, personal communication).

A similar strategy will likely be necessary for the production of rapeseed with greater erucic acid levels in seed oil. If accumulated at greater than 90% of the seed oil content, erucic acid would compete more favorably with petrochemicals used in the production of nylon 13,13. Erucic acid also serves as a slip agent in plastic film production (1). Due to the specificity of the native LPAAT in rapeseed, erucic acid is excluded from the *sn*-2 position of TAG. This limits the maximum erucic acid content to around 60% in high erucic acid rapeseed (HEAR) lines (46). Recently, cDNA clones encoding erucoyl CoA-specific LPAAT from *Limnanthes douglasii* were isolated (47). Introduction of this gene into HEAR varieties should increase the erucic acid levels in TAG. Further metabolic modification might be necessary to maximize erucic acid production. The reduction of native LPAAT enzyme levels through anti-sense engineering combined with the increase of the FAE levels through sense reintroduction, along with the introduction of the erucoyl-specific LPAAT into HEAR varieties, would likely maximize erucic acid production.

PRODUCTION OF OXYGENATED FA

As described previously, most of the FA found in plant tissues are comprised of five major types, the saturated 16-carbon and 18-carbon FA and the mono-, di-, and triunsaturated 18-carbon FA. Also, as mentioned earlier in this review, altered thioesterase or elongase activities can result in accumulation of FA of different chain lengths. In addition to the control of differing degrees of FA unsaturation and chain lengths, plants have the capacity to produce a wide variety of FA derivatives. The plant kingdom has been found to contain more than 200 different FA (48), and this list is likely to grow as additional plant species are examined. Most of these "unusual" FA only accumulate in the triglycerides of seed oils. The biological roles of most of these "unusual" FA are not known, but it is thought that many or most of them may have a role in defense against pests, as do many secondary products in plants. Here we will focus only on some of the oxygenated FA derivatives. The formation of these oxygenated FA derivatives in plant tissues is outlined in Scheme 1 (FA esterified to phospholipids, especially PC, can be converted by epoxygenase or hydroxylase into epoxy or hydroxy derivatives. FA also can be converted by lipoxygenase into hydroperoxy derivatives. Most lipoxygenases are thought to act on FA which are first "released" by phospholipase or lipolytic acyl hydrolase before peroxidation. Lipoxygenase can peroxidize linoleic or linolenic acids forming hydroperoxy linoleic or linolenic acids. The primary products of lipoxygenase, the FA hydroperoxides usually do not accumulate in plant tissues and can be rapidly converted into additional products by one of three known pathways. Most plant tissues appear to have the hydroperoxide lyase and allene oxide synthase branches of the lipoxygenase pathway. To date, peroxygenases have only been demonstrated in seedlings of some plants. Hydroperoxide lyase can cleave FA hydroperoxides forming 6- and 12-carbon aldehydes. Many plants have the



SCHEME 1

capacity to convert the 6-carbon aldehydes into alcohols. Allene oxide synthase converts the FA hydroperoxides into an unstable epoxy intermediate, which can be converted into a cyclic ketone by a cyclase. A series of subsequent reactions can result in formation of jasmonic acid, which can be converted into methyl jasmonate. Peroxygenases convert fatty acid hydroperoxides into epoxides which can be converted to hydroxides by hydroxylases.)

Hydroxylases and epoxygenases. Transformation of plants with heterologous genes from other organisms may be a way to produce entirely new novel compounds previously not seen in those plants. Often these genes come from noncrop plants or from plants that are not suitable for particular agricultural regions. By putting these genes into agriculturally valuable crops to modify lipid synthesis, we can essentially create new forms of currently high yielding plants. This will allow us to use the plants as chemical factories, accumulating potentially valuable compounds. Epoxy and hydroxy FA are instances of high-value FA derivatives.

An example of a hydroxy FA which can accumulate in the storage oil of higher plant seeds is 12*R*-hydroxyoctadec-*cis*-9-enoic acid (ricinoleic acid). This FA has been found in at least a dozen genera of ten different higher plant families (48). One species which accumulates ricinoleic acid in the seed oil is the castor oil plant (*Ricinus communis* L.). The castor oil plant does not have agronomic characteristics such that it can be grown commercially on a large scale. Thus there has been interest in cloning the hydroxylase gene responsible for hydroxy FA synthesis in order to genetically engineer more productive oilseeds for hydroxy FA accumulation. There has been little

biochemical information characterizing the enzymes that are involved in hydroxy FA synthesis. This is apparently due to the fact that hydroxylase, such as that in developing castor endosperm, is labile and associated with the endoplasmic reticulum (49,50). Early *in vivo* labelling experiments indicated that the ricinoleic acid of maturing castor endosperm is synthesized *via* direct hydroxylation of an oleic acid precursor (51). This microsomal hydroxylase apparently requires oleoylphosphatidylcholine, O_2, and NADH. Also, iron appears to be involved in the catalysis of this enzyme, but this enzyme is not a cytochrome P-450 protein (49,50). These characteristics of the castor hydroxlyase indicated a superficial resemblance to ω6 FA desaturases (52,53) and led to the development of a successful strategy for cloning of the oleate 12-hydroxylase from developing castor oil endosperm. Expression of a full-length putative hydroxylase cDNA clone in transgenic tobacco caused the accumulation of low levels of ricinoleic acid in the seed, indicating that they had indeed cloned an oleate 12-hydroxylase. The poor accumulation of ricinoleic acid in the transgenic tobacco seed indicated to van de Loo *et al.* (52) that other enzymes, such as acyltransferases with specificity for transfer of hydroxy FA into TAG, are needed.

Epoxides are valuable for the production of several industrial products and are currently chemically synthesized at some expense. Engineering productive oilseeds with enzymes responsible for epoxide synthesis could potentially allow us to have an abundant renewable source of these valuable chemicals. Genetic engineering of oilseeds for epoxy FA accumulation would increase the value of the oil of such modified seeds for industrial uses. A considerable market currently exists for epoxy FA, particularly for epoxy coatings and plasticizers. The current United States plasticizer market is about two billion pounds per year (54). Presently most of this is derived from petroleum. In addition, there is industrial interest in the use of epoxy FA in oil-based paints, lubricants, and lubricant additives, adhesives, insecticides and insect repellants, crop oil concentrates, and the formulation of carriers for slow-release pesticides and herbicides (55,56). Also, epoxy FA can readily and economically be converted to hydroxy FA and their derivatives, which are useful starting materials for the production of plastics, as well as for detergents and lubricants. Examples of plastics which can be produced from hydroxy FA are polyurethanes and polyesters (57). If a soybean and/or other major oilseed were developed which accumulated epoxy FA, it is likely that other valuable products would be developed using this as an industrial chemical feedstock in the future.

Epoxidized oil would likely be more economical if the biosynthetic reactions in oilseed crops, such as soybean, were altered such that the seeds themselves converted the polyunsaturated FA into epoxy FA. There is no known way to produce a soybean which accumulates epoxy FA by conventional breeding and genetics. However, certain genotypes of several plant species accumulate high levels of epoxy FA in the seed oil. Epoxy FA, like vernolic (*cis*-12,13-epoxyoctadeca-*cis*-9-enoic) and coronaric (*cis*-9,10-epoxyoctadeca-*cis*-12-enoic) acids, have been found as a component of the seed oil of species represented by a number of plant families, such as Compositae, Euphorbiacea, Onagraceae, Dipsacaceae, and Valerianacea (58). Plant epoxygenases are known which transform unsaturated FA into epoxy FA (59,60). The best examples of epoxy FA accumulating plants are *Vernonia galamensis* and *Euphorbia lagascae,* both of which accumulate vernolic acid (55,61,62). Some accessions of *V. galamensis* have as much as 40% oil, which is comprised of 80% vernolic acid (63). Although soybeans do not accumulate large quantities of epoxy FA in their oil, they are known to contain the requisite enzymes for producing epoxy FA from unsaturated FA. Soybean seedlings have been shown to contain an enzyme known as peroxygenase that was shown to epoxidize either oleic or linoleic acid using a hydroperoxy oxygen of hydroperoxylinoleic acid (64). A similar enzyme has been found in broad bean (65). In addition to utilizing hydroperoxylinoleic acid as a source of oxidation potential, the broad bean enzyme was shown to be capable of utilizing H_2O_2 (66) and cumene hydroperoxide in the epoxidation process (65). Peroxygenase and related reactions will be discussed further in the following section.

Although major oilseeds such as soybeans clearly have the enzymes, such as lipoxygenase and peroxygenase required to produce epoxy FA, the process by which the seeds of certain species of *Vernonia* and *Euphorbia* accumulate epoxytriglycerides appears to be due to an enzyme not present in major commercial oilseeds such as soybeans. The production of epoxy FA and derivatives in defense responses appears to be widespread in plants (see below), but the accumulation in storage oil of certain seeds such as *Vernonia* and *Euphorbia* appears to be due to a different, much less common mechanism (67). Biochemical studies by Bafor *et al.* (62) indicate that developing seeds of these plants contain an enzyme known as an epoxygenase which converts linoleic acid into vernolic acid in a one-step reaction. Most epoxygenation reactions in biological systems appear to be catalyzed by cytochrome P-450 monooxygenase enzymes. This epoxygenase also appears to be a cytochrome P-450 monooxygenase (62).

Epoxy and hydroxy FA are apparently formed in plant defense reactions (68) but *via* a different mechanism than the accumulation in the oilseeds mentioned previously. These compounds can have antimicrobial properties and also can provide monomers for cutin synthesis. Cutin is the insoluble polymeric structural component of the cuticle of all aerial parts of plants except periderms (69,70). Cutin monomers include epoxy, monohydroxy, epoxy-hydroxy, and dihydroxy 16- and 18-carbon FA. Blée and Schuber (71) provided good evidence that the formation of these cutin monomers, at least in wound-induced cutin formation, involves epoxidation of oleic acid by peroxygenase using lipoxygenase-derived hydroperoxides. The epoxide is hydrolyzed to the corresponding diol by hydrolase and the ω-hydroxylation most probably is catalyzed by a cytochrome P450. The ω-hydroxy 16- and 18-carbon FA, which are the major aliphatic monomer components of suberin, might be formed

via the same mechanism as the ω-hydroxy FA involved in cutin formation. Cutin and suberin formation can be formed in plant pest defense reactions as a physical barrier to invading organisms (68,72).

Lipoxygenase (LOX) and related enzymes. LOX are ubiquitous plant enzymes that catalyze the peroxidation of *cis,cis*-1,4-pentadiene moieties of FA such as 18:2 and 18:3. LOX can affect the quality of vegetable oils by initiating FA peroxidation prior to oil extraction. Many plants have been shown to contain multiple LOX isozymes encoded by different genes. Table 1 shows the wide range of cloned LOX from 11 different plants. It is estimated that there are up to 12 different LOX genes expressed at different times in the lifecycle of the soybean (73–76). LOX 1–3 are the major isozymes expressed in cotyledons and embryonic axes seen from the maturation phase of seed development until maturity, but they are not expressed in seedcoats (75). Preliminary evidence indicates that a minor isozyme, different from LOX 1–3, is constitutively expressed in most or all tissues. The complete coding regions of all three soybean embryo LOX cDNAs have been sequenced (77–80), and LOX from a variety of other plants also have been sequenced in recent years (Table 1). All three soybean seed LOX show a high degree of homology, at least 70% at the amino acid sequence level (78), with LOX 1 and 2 being particularly homologous [81% at the amino acid sequence level (Refs. 78,81)]. Plant LOX have been divided into two major groups, LOX1 and 2, based on general homologies (82). Most plant LOX are of the LOX1 class, an example of which is soybean LOX1. There are two representatives known for the LOX2 class, both of which have putative plastid transit sequences, one from rice and one from *Arabidopsis* (83,84). Interestingly, the other known *Arabidopsis* LOX is a LOX1 type (82,85). Recently, Bell *et al.* (86) provided good evidence that the type 2 LOX of *Arabidopsis* is chloroplast localized.

The FA hydroperoxides resulting from LOX action in higher plants, including soybeans are metabolized by one of several major pathways operative in plant tissues (Scheme 1; Refs. 64,68,76,87–90). In one pathway, hydroperoxide lyase catalyzes the cleavage of 13-hydroperoxylinoleic or linolenic acid into the 12-carbon compound, 12-oxo-*cis*-9-dodecenoic acid, and the 6-carbon aldehydes, hexanal or *cis*-3-hexenal. The C_6 aldehydes have distinct odors or aromas. Hexanal is undesirable in most food applications, but *cis*-3-hexenal and *trans*-2-hexenal are often desirable. Hexanal and hexenals are frequently converted to their corresponding alcohols by alcohol dehydrogenase, and the alcohols can be converted into esters. Many of these alcohols and esters are also quite aromatic and important in the flavor of many fruits and vegetables including in the so-called "fresh notes."

Soybean seeds have high 6-carbon aldehyde production capacity, but this often appears to be limited by free FA availability (91,92) and is dependent on formation of 13-hydroperoxy products by LOX and/or the selectivity of hydroperoxide lyases. Some LOX form 9-hydroperoxy derivatives in plants, which can be converted into 9-carbon aldehydes in some plant tissues such as cucumber, melons, and some other fruit (93–96).

The predominant FA in soybean seeds is linoleic acid. Hence the main LOX/hydroperoxide lyase product in soybean seeds is the saturated aldehyde, hexanal. Hexanal is undesirable in most food applications using soybean seed, contributing a rancid odor (97). Also, as mentioned previously, LOX may reduce vegetable oil stability. Soybean seeds are especially rich in LOX, and breeders have long been interested in the genetic reduction of LOX activity of soybean seed. LOX null mutants lacking LOX1–3 have been produced by traditional plant breeding methods (98,99), but so far no transgenic plants have been produced with LOX anti-sense insertions. The hydroperoxide lyase protein has been previously purified from green tea (*Thea sinensis)* (100) and recently characterized (101), but the gene has not yet been isolated. Bell pepper hydroperoxide lyase is a 55 kD heme protein resembling the cytochrome P450 (102), allene oxide synthase, from flaxseed (Refs. 103,104; see Table 1 and following text). The hydroperoxide lyase does not show a spectral change upon carbon monoxide treatment characteristic of cytochrome P450, but it may still be an unusual type of cytochrome P450. Due to their wide distribution among plant species and tissues and role in volatile, odiferous compound generation, LOX/hydroperoxide lyase can significantly impact quality of many foods based on or containing plant products.

A second pathway for metabolism of hydroperoxy FA (at least for linolenic acid) in plants is the conversion to an allene oxide by allene oxide synthase (also known as hydroperoxide dehydratase) (103–105). The allene oxide can undergo hydrolysis resulting in the formation of ketols. Alternatively, the allene oxide can undergo rearrangement and cyclization resulting in the formation of 12-oxo-phytodienoic acid (103). The 12-oxo-phytodienoic acid can be converted by a series of reactions involving a reductase and β-oxidations into jasmonic acid (88). There has been considerable interest in the physiological role of jasmonates. Jasmonic acid and related metabolites appear to be important signal molecules in plant storage product accumulation and response to stress, such as a water deficit or pest attack. Hamberg and Gardner (89), Sembdner and Parthier (106), Parthier (107), and Reinbothe *et al.* (108) recently reviewed this subject. Jasmonic acid has been shown to occur in many plant tissues including soybeans, especially in immature pericarp (109). Simpson and Gardner (105) examined the activity of the first two enzymes of the jasmonic pathway in soybean tissues. The highest activity was found in seedcoats followed by pericarp of immature fruit. This is consistent with studies on levels of jasmonic acid in soybean tissues (109). A cDNA encoding the allene oxide synthase from flaxseed has been cloned (104). Transgenic potato plants have been produced using this flax allene oxide synthase cDNA, which have 6- to 12-fold higher levels of jasmonic acid than nontransformed control plants (110).

The subcellular location of oxylipin pathway enzymes has not been clearly elucidated. It has been postulated that putative phospholipases thought to be involved in oxylipin for-

mation might be plasma membrane-located (111), but some studies indicate that this is not always the case (112). Most LOX isozymes in plant tissues appear to be cytoplasmically located but, as mentioned before, LOX of the 2 class appear to be located in plastids (84,86). Hydroperoxide lyase and allene oxide synthase also appear to be plastid located (59,110,113,114).

Peroxygenase is a unique membrane-associated oxygenase, occurring in soybean seedlings and other higher plant tissues, which catalyzes the hydroperoxide-dependent epoxidation of mono- and polyunsaturated FA (115,116). In developing seeds of *E. lagascae,* both peroxygenase and a cytochrome P-450 epoxygenase activity can be found, but in germinating seedlings only peroxygenase activity is detectable (67). Unlike the cytochrome P450 epoxygenase from *E. lagascae*, the peroxygenase is not a P450 oxygenase. In the peroxygenase reaction, the hydroperoxide is converted into hydroxy and hydroxy–epoxy FA (67,90). Soybean seedlings contain an enzyme, epoxide hydrolase, capable of hydrating the epoxide products of peroxygenase into their corresponding diols (117–119). The soybean FA epoxide hydrolase protein has been purified (117), and this will allow the isolation of the DNA clone with future work. Epoxy FA derivatives have been shown to induce resistance to pathogens in rice (120). The involvement of LOX/peroxygenase/epoxide hydrolase in a cascade is thought to be a defense reaction in plants in response to pathogen infection (115).

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ARTICLE

Characterization of Oleoyl-12-Hydroxylase in Castor Microsomes Using the Putative Substrate, 1-Acyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine

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ABSTRACT: We have characterized the oleoyl-12-hydroxylase in the microsomal fraction of immature castor bean using the putative substrate, 1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine (2-oleoyl-PC). Previous characterizations of this enzyme used oleoyl-CoA as substrate and relied on the enzyme transferring oleate from oleoyl-CoA to lysophosphatidylcholine to form 2-oleoyl-PC (acyl-CoA:lysophosphatidylcholine acyltransferase) in addition to oleoyl-12-hydroxylase. The present assay system and characterization use 2-oleoyl-PC as substrate (oleoyl-12-hydroxylase alone). Use of the actual substrate for assay purposes is important for the eventual purification of the oleoyl-12-hydroxylase. Ricinoleate (product of oleoyl-12-hydroxylase) and linoleate (product of oleoyl-12-desaturase) were identified as metabolites of oleate of 2-oleoyl-PC by high-performance liquid chromatography and gas chromatography/mass spectrometry. The activity of oleoyl-12-hydroxylase in the microsomal fraction reached a peak about 44 d after anthesis of castor, while the activity of oleoyl-12-desaturase reached a peak about 23 d after anthesis. The optimal temperature for the oleoyl-12-hydroxylase was about 22.5°C, and the optimal pH was 6.3. Catalase stimulated oleoyl-12-hydroxylase while bovine serum albumin and CoA did not activate oleoyl-12-hydroxylase. The phosphatidylcholine analogue, oleoyloxyethyl phosphocholine, inhibited the activity of oleoyl-12-hydroxylase. These results further support the hypothesis that the actual subtrate of oleoyl-12-hydroxylase is 2-oleoyl-PC.

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Castor plants (*Ricinus communis* L.) have long been cultivated and harvested for production of oil. Castor oil obtained from the seeds is unique in that ricinoleic acid [D(+)-12-hydroxy-*cis*-9-octadecenoic acid] comprises roughly 90% of its fatty acids, and triricinolein makes up 70% of its triglycerides (1). Ricinoleic acid has many industrial uses, including the manufacture of lubricating oils, paints, coatings, cosmetics, thermopolymers, and other plastics. However, castor bean contains a toxic albumin, ricin, and protein polysaccharides that are potent allergens (2). Because of the potential danger of these components in harvest and processing, it is desirable to produce ricinoleic acid in an oilseed lacking these components.

Research on the enzymology of oleoyl-12-hydroxylase began nearly 30 years ago. The studies showed that the enzyme catalyzes the hydroxylation of oleoyl-CoA to ricinoleic acid in a reaction requiring oxygen and NADH, and has the properties of a microsomal mixed function oxidase (3). Ricinoleic acid is synthesized by direct hydroxy substitution at the 12-position of oleic acid with retention of configuration at the 12-position, and the hydrogen lost is of the D-configuration (4). The enzyme, which has not been purified yet, is located in the endoplasmic reticulum (microsomal) fraction (5). The hydroxylation requires reduced cytochrome b_5. Cytochrome P-450 is not involved (6), although many hydroxylation reactions in living systems involve cytochrome P-450. The immediate substrate for the oleoyl-12-hydroxylase has been suggested to be 1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine (2-oleoyl-PC) based on rapid incorporation of [^{14}C]oleate from oleoyl-CoA into phosphatidylcholine (PC) (7,8). We report here the characterization of oleoyl-12-hydroxylase using 2-oleoyl-PC as substrate. Recently, the castor oleoyl-12-hydroxylase was cloned; however, expression in the seed of transgenic tobacco was very low (9). Our ultimate goal is to develop a transgenic plant, producing ricinoleate in large quantity. For this purpose, the biosynthetic pathway of ricinoleate and triricinolein, and the enzymes involved, must be better understood to maximize the expression. Since oleoyl-12-hydroxylase gene has been cloned, we should try to understand the regulation of the enzyme, oleoyl-12-hydroxylase.

EXPERIMENTAL PROCEDURES

Plant material and chemical. Castor plants (*R. communis* L., United States Department of Agriculture accession 215679) were grown in a greenhouse. Harvested immature fruits were cut in half, and the endosperm was excised and used for the preparation of microsomes. Removed endosperm was immediately frozen in liquid nitrogen and stored in a freezer at –80°C until used for microsomal preparation. The microsomal

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Abbreviations: BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; OEPC, oleoyloxyethyl phosphocholine; 2-oleoyl-PC, 1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; TLC, thin-layer chromatography.

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fractions used to measure enzyme activity and protein concentration vs. maturity of the bean were prepared from whole beans except the shells. Oleoyloxyethyl phosphocholine (OEPC) was purchased from Calbiochem (La Jolla, CA).

Microsomal preparation. Microsomes were prepared in a cold room (4°C) using a procedure similar to that of Bafor *et al.* (7). The endosperms (about 8 g) were ground in a mortar with 5 parts (vol/wt) of extraction buffer, 0.1 M sodium phosphate pH 6.3, containing 0.1% bovine serum albumin (BSA), 0.33 M sucrose, and 1000 units of catalase/mL. The homogenate was filtered through two layers of Miracloth and a layer of cheesecloth. The residue was ground again with extraction buffer and filtered. The total volume of extraction buffer used was 80 mL. The combined filtrates were centrifuged at 10,000 × *g* for 20 min using four tubes. The resulting supernatant was filtered through Miracloth and centrifuged at 105,000 × *g* for 90 min in eight 10-mL tubes. The fat pads and supernatants were removed and the pellets were suspended in the buffer, 0.1 M sodium phosphate pH 6.3, containing 0.1% BSA, and 1000 units of catalase/mL by repeated pipetting into a disposable plastic pipette with a very small opening (about 0.75 mm) on the tip. The volume of the buffer used was 1 mL/g of starting material. After the pellet was uniformly suspended, it was aliquoted and frozen in liquid nitrogen and then stored at −80°C. Protein concentration was determined by the bicinchoninic acid method (10).

Microsomal incubation. The microsomal incubation mixture included, in general, in a total volume of 1 mL, sodium phosphate (0.1 M, pH 6.3), NADH (0.5 μmol), ATP (0.5 μmol), $MgCl_2$ (0.5 μmol), catalase (1000 units), the microsomal fraction from castor bean (10 μL, about 92 μg of protein), and 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (0.125 μCi, 2.16 nmol, 58.0 mCi/mmol; Amersham, Arlington, IL) in 20 μL of ethanol. The radioactive subtrate in 20 μL of ethanol was added last into a screw-capped tube followed by immediate mixing. The incubation mixture in the tube was then incubated in a shaking water bath (Dubnoff metabolic shaking incubator; Precision Scientific, Chicago, IL) for 30 min at 22.5°C. The incubation was stopped by addition of 3.75 mL of chloroform/methanol (1:2, vol/vol). Duplicate incubations were done under each incubation condition with variations shown in Figures 2, 4–10.

Extraction and hydrolysis of lipids. After the incubation was stopped, the mixture was again mixed with 0.63 mL of chloroform and 0.63 mL of water. The upper layer was removed by a Pasteur pipette. The lower lipid phase was dried by evaporator under a stream of nitrogen and then hydrolyzed and methylated in 5% HCl/methanol (1 mL) at 80°C for one hour. The fatty acid methyl esters formed were extracted with 2 × 1 mL of hexane.

High-performance liquid chromatography (HPLC) on short column. HPLC of the methyl esters was carried out on a liquid chromatograph (Waters Associates, Milford, MA) consisting of two pumps (M510), a multiwavelength detector (M490), and a chromatography manager (Millenium 2010). For the purpose of quantifying the radioactivities of the methyl esters of ricinoleate, linoleate, and oleate, a short reversed-phase C_{18} column (5 cm × 0.46 cm, 3 μm, Microsorb-MV; Rainin, Woburn, MA) was used to save time. The eluent was delivered at a linear gradient ranging from 90 to 100% methanol in 15 min at a flow rate of 1 mL/min for 10 min. The methyl esters of ricinoleate (1.5 min), linoleate (4.8 min), and oleate (6.7 min) were detected at 205 nm and were well separated (Fig. 1). The radioactivities of these methyl esters were quantified by a radioactivity flow detector (Flow-one, Model CR; Packard, Downers Grove, IL). The radioactivity flow detector included a flow cell of 0.5 mL, with delay time of 30 s comparing with the multiwavelength detector. The flow rate of liquid scintillation fluid (Ready Safe; Beckman Instruments Inc., Fullerton, CA) used by the radioactivity flow detector was 3.0 mL/min. The enzyme activities were calculated from the radioactivity percentage conversion of ricinoleate and linoleate.

HPLC on long column. The metabolites, radiolabelled ricinoleate and linoleate, were identified by the HPLC of the fatty acid methyl esters using a long reversed-phase C_{18} col-

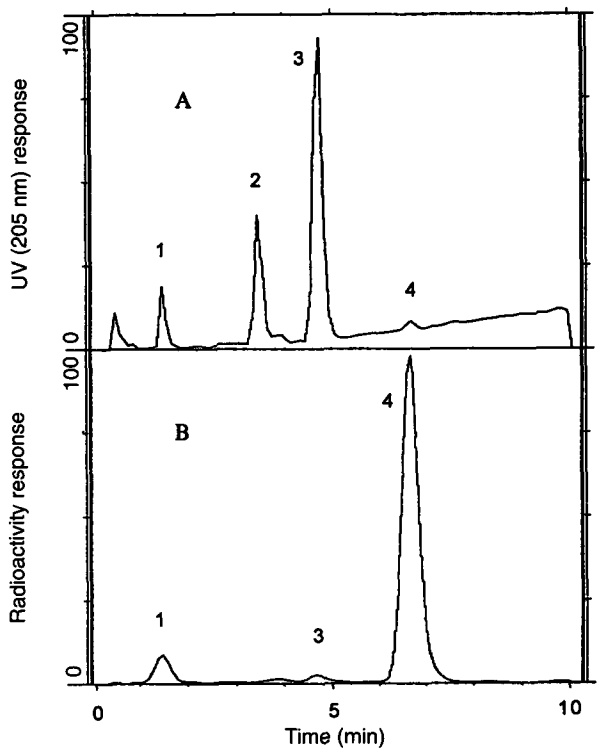


FIG. 1. Typical high-performance liquid chromatography (HPLC) radiochromatogram of the incubation products after methanolysis (produced methyl esters of fatty acids). For HPLC conditions, see HPLC on short column in the Experimental Procedures section. A: chromatogram detected by ultraviolet (UV) at 205 nm; B: chromatogram detected by radioactivity flow detector; 1: methyl ester of ricinoleic acid (1.5 min), 2: methyl ester of linolenic acid (3.5 min), 3: methyl ester of linoleic acid (4.8 min), 4: methyl ester of oleic acid (6.7 min). Minor radioactive peak (chromatogram B) at retention time 3.9 min was unknown and was also shown in the incubation without microsomal fraction. The fatty acids shown in chromatogram A were endogenous in the microsomal fraction.

umn (25 cm × 0.46 cm, 5 μm, Ultrasphere ODS; Beckman Instruments Inc.) (11). The eluent used was delivered at a linear gradient ranging from 90 to 100% methanol in 40 min at a flow rate of 1 mL/min. The retention times for the methyl esters were 7.87 min for ricinoleic acid, 21.52 min for linoleic acid, and 27.02 min for oleic acid. Samples were identified by cochromatography with standards and by matching the retention times of the standards from the ultraviolet detector and the radioactivity flow detector. The radiolabelled methyl esters of ricinoleate and linoleate were also identified by gas chromatography/mass spectrometry (12) of the HPLC fraction of the radioactive peaks.

Analysis of intact lipids. The lipid extracts from the microsomal incubations were dissolved in 2 mL chloroform/acetic acid (100:1, vol/vol) and fractionated on Sep-Pak silica columns (Waters Associates) into neutral lipid, glycolipid, and phospholipid fractions by the method of Lynch and Steponkus (13). Phospholipid and neutral lipid separations by thin-layer chromatography (TLC) have been described (14). Phospholipids were separated by TLC on silica gel plates (EM Separations Technology, Gibbstown, NJ) which were first immersed in 100 mM ammonium sulfate and dried for 1.5 h at 120°C. Phospholipids were applied to the plates in a spotting chamber. Separation was in the solvent system 1-propanol/chloroform/propanoic acid/0.1% (wt/vol) KCl, (3:2:2:1, by vol). Bands containing lipids to be analyzed were routinely localized by iodine staining. Lipids were identified by comigration with standards, and for phospholipids, by staining with molybdenum blue reagent (Sigma, St. Louis, MO). Lipid bands were removed from the plates by scraping, and counts in lipid bands were determined by liquid scintillation counter.

RESULTS AND DISCUSSION

In this report, we present the first characterization of oleoyl-12-hydroxylase in the microsomal fraction of immature castor bean using the putative substrate, 2-oleoyl-PC. Previous characterizations of this enzyme (3,7,8) used oleoyl-CoA as substrate and relied on the prior enzyme transferring oleate from oleoyl-CoA to lysoPC to form 2-oleoyl-PC (acyl-CoA:lysoPC acyltransferase) in addition to oleoyl-12-hydroxylase. The present assay system and characterization use oleoyl-PC as substrate (oleoyl-12-hydroxylase alone). Use of the actual substrate for assay purposes is important for the eventual purification of the oleoyl-12-hydroxylase, when it is separated from the acyl-CoA:lysoPC acyltransferase.

HPLC with ultraviolet detector and radioactive flow detector is an effective method to identify and quantify radioactive oleate, ricinoleate, and linoleate. A typical radiochromatogram is shown as Figure 1. The incubation of the microsomal fraction from castor bean with 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-PC followed by the methanolysis of incubation products (produced methyl esters of fatty acids) showed the presence of radiolabelled ricinoleate (due to oleoyl-12-hydroxylase) and linoleate (due to oleoyl-12-desaturase) by HPLC. Radiolabelled linolenate (due to the further desaturation of linoleate) was not detected (Fig. 1). We believe that the enzyme is active on *sn*-2 position, but we cannot rule out the possibility that [1-^{14}C]oleate at the *sn*-2 position of PC is incorporated into ricinoleate and linoleate after reacylation. The reacylation problem may be avoided by using the ether analog as substrate (15). The enzymes in the microsomal fraction remained active at −80°C for at least eight months. Radiolabelled oleoyl-CoA has previously been used as substrate in most studies of oleoyl-12-hydroxylase (3,7,8). Also, microsomal membranes labelled *in situ* with 1-acyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-PC were used recently (6). The latter was made by the incubation of microsomes with [^{14}C]oleoyl-CoA, and only 71% of [^{14}C]oleate was in position 2 of PC, which was the presumed substrate of oleoyl-12-hydroxylase. The characterization of oleoyl-12-hydroxylase using this microsomal substrate has not been reported (6). In the present study, we used commercially available 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-PC, and the activity was more than twice that using microsomal membranes labelled *in situ* with 1-acyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-PC. The radioactive substrate, 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-PC, dissolved in 20 μL of ethanol, was added to the other incubation components, followed immediately by mixing the incubation mixture before the incubation. The addition of the substrate dissolved in ethanol was the most effective way to suspend the substrate based on availability for the enzymic reaction. Diethyleneglycol monoethyl ether and detergent (TX-114) as substitutes for ethanol to suspend the substrate gave inferior results, as activity was much lower. It seems that ethanol can deliver the substrate more effectively to the active site of the oleoyl-12-hydroxylase, with minimal inactivation at this final ethanol concentration (2%). We previously have used ethanol instead of commonly used detergents to deliver lipid substrate to the enzyme (16). The amount of ethanol used should be minimal to minimize the inactivation and large enough to deliver lipid substrate quantitatively and accurately. We chose to use 20 μL of ethanol. Other alcohols were not tried since ethanol was effective.

The activities of oleoyl-12-hydroxylase and oleoyl-12-desaturase in developing castor bean are dependent on the maturity of the bean as shown in Figure 2. At about 16 d after anthesis, no oleoyl-12-hydroxylase or oleoyl-12-desaturase activity was detected. At this developmental stage, the seed coat (shell) was soft and white or very light green. The endosperm occupied only about 5% of the seed volume. The highest activity of oleoyl-12-desaturase was seen at about 23 d after anthesis. At this developmental stage, endosperm filled only about 15% of the shell. The shell was soft and light green or white, and less than10% of the seedcoat area was violet. As the bean developed, the violet spot enlarged, darkened, and hardened to eventually cover the whole shell. The highest activity of oleoyl-12-hydroxylase was seen at about 44 d after anthesis. At this developmental stage, endosperm almost filled the shell (95%), and the shell just turned dark brown and hard without any area of soft and light color remaining. The enzyme activities decreased but remained measurable

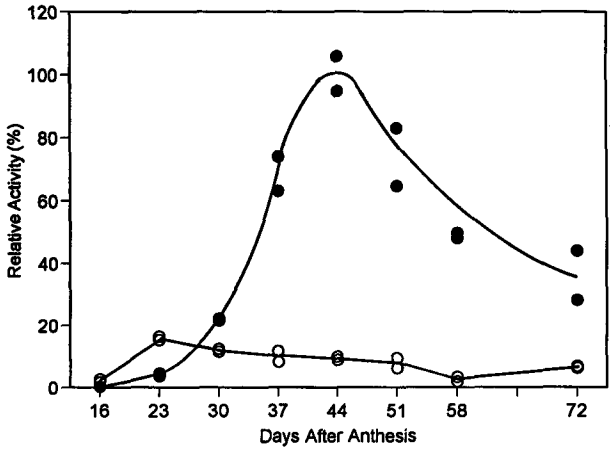


FIG. 2. Relative activities of oleoyl-12-hydroxylase (●) and oleoyl-12-desaturase (○) in the castor microsomal fractions at various times after anthesis. Circles represent individual determinations.

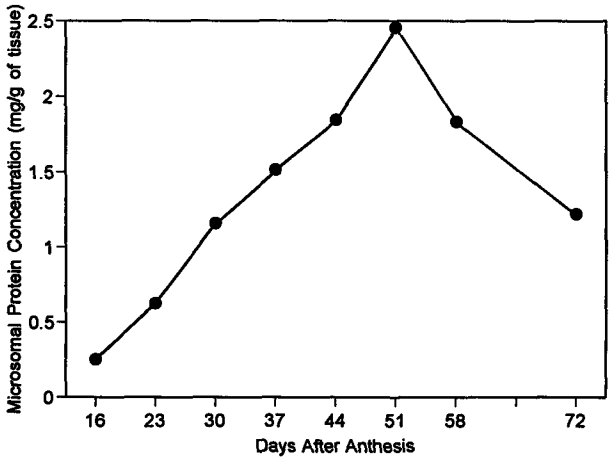


FIG. 3. Protein concentrations in the castor microsomal fraction vs. time after anthesis. Circles represent individual determinations.

until the capsule became dry and brown. The peak of activity of oleoyl-12-desaturase in castor bean development was thus much earlier than that of oleoyl-12-hydroxylase. The activity of oleoyl-12-hydroxylase at the later developmental stages was much higher than that of oleoyl-12-desaturase (Fig. 2). Since the substrates of both enzymes are the same, the activity difference of these two enzymes in the later developmental stage is obviously one of the reasons castor oil is composed of 85–90% ricinoleate. This is the first study that has simultaneously monitored and compared the activities of the oleoyl-12-hydroxylase and oleoyl-12-desaturase (two enzymes that share a common substrate and have other properties in common) in developing castor bean. Oleoyl-12-hydroxylase has not been purified yet, but it has been shown to be a member of the membrane-bound desaturase family based on DNA sequence (9); however the oleoyl-12-desaturase in spinach chloroplast has been purified and the gene cloned (17). Microsomal oleoyl-12-desaturase reported here desaturates PC, and the electron donor has been identified as reduced cytochrome b_5 (6) while chloroplast oleoyl-12-desaturase desaturates a variety of substrates including free oleic acid, free erucic acid, 1-oleoyl-*sn*-glycerol 3-phosphate, and galactolipids; the electron donor has been identified as reduced ferredoxin (18). Protein concentration in the microsomal fraction was highest at about 51 d after anthesis as is shown in Figure 3. At this developmental stage, the endosperm just filled the seed inside the shell completely.

The amount of the microsomal fraction affects the activity of oleoyl-12-hydroxylase as is shown in Figure 4. We chose 10 µL of microsomal fraction (equivalent to 92 µg of microsomal protein) for our routine incubations, and the activity was about half of the maximum activity shown in Figure 4. The higher specific activity at the lower microsome concentration (shown in Fig. 4) might be due to the fact that the concentration of the unlabelled endogenous substrate in the microsomal fraction was also reduced. The radioactive substrate thus had less competition probably from the endogenous substrate in the incubation. The amounts of the microsomal fraction used were much lower than used in previous studies (300 µg–1 mg) (3,6,7) in which radioactive oleoyl-CoA was used as substrate. Interestingly, the conversion of oleoyl-CoA into ricinoleate (McKeon, T.A., Lin, J.T., and Stafford, A.E., unpublished data) in the microsomal fraction was about eight times that of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-PC. It seems that oleoyl-CoA gets incorporated into the PC pool more readily than exogenous PC.

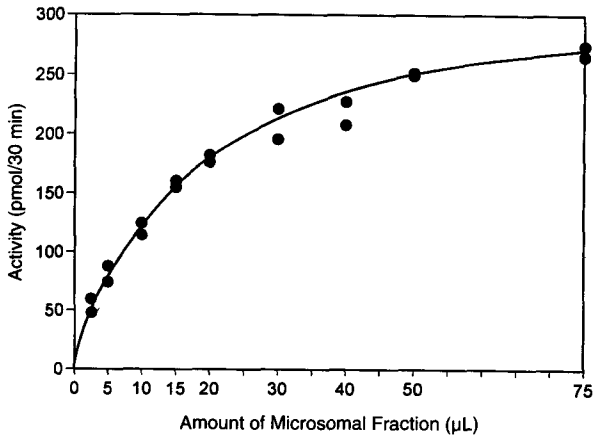


FIG. 4. Effect of various amounts of microsomal fraction on the activity of oleoyl-12-hydroxylase. Protein concentration of microsomal fraction was 9.2 µg/µL.

The rationale for using 2-oleoyl-PC as the substrate is twofold: to use the actual substrate in incubations and to eliminate the need for extraneous enzymes such as the acyl-CoA:lysoPC acyltransferase in the incubations. It was important, then, to optimize incubation conditions using 2-oleoyl-PC as the substrate. We therefore examined oleoyl-12-hy-

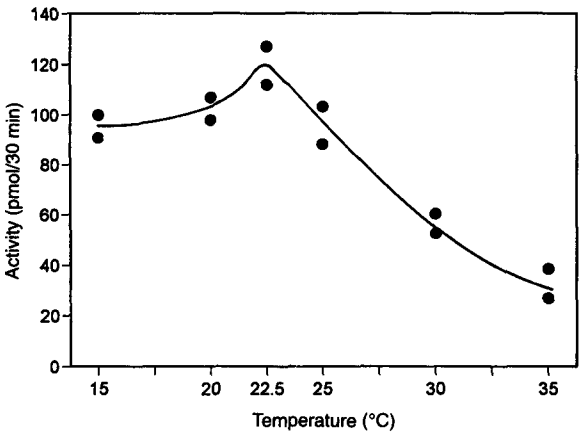


FIG. 5. Effect of temperature on the activity of oleoyl-12-hydroxylase in the microsomal fraction of castor bean.

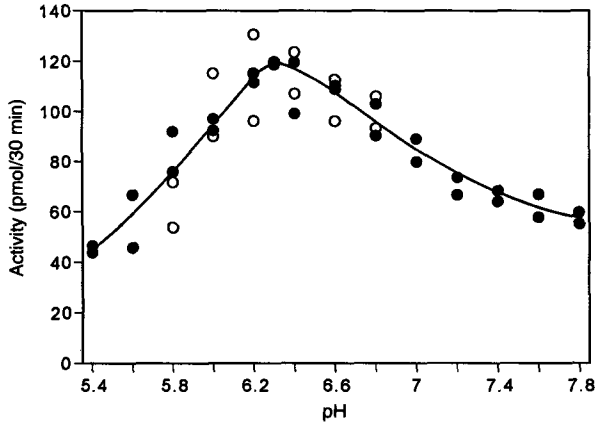


FIG. 7. Effect of pH on the activity of oleoyl-12-hydroxylase in the microsomal fraction of castor bean. The activities using phosphate buffer (0.1M) are represented by ● (with best-fit curve), while the activities using 2[*N*-morpholino]ethanesulfonic acid buffer (0.1M) are represented by ○.

droxylase activity as a function of temperature, incubation time, and pH, and we examined the effect of various additions routinely (3,7,8) added to hydroxylase incubations. Figure 5 shows the activities of oleoyl-12-hydroxylase at various temperatures. The maximum activity was observed at 22.5°C and was therefore used in our routine incubations. A temperature optimum has not been reported for the oleoyl-12-hydroxylase when oleoyl-CoA was used as substrate. Figure 6 shows the efficiency of conversion of oleate to ricinoleate for various incubation times. To obtain sufficent radioactive ricinoleate for analyses, 30-min incubations were used. The activity of oleoyl-12-hydroxylase was also dependent on pH, as is shown in Figure 7. The optimal activity was at pH 6.3 when sodium phosphate buffer, 0.1 M, was used. The same pH optimum for activity and the same maximal activity were seen using 2-[*N*-morpholino]ethanesulfonic acid buffer. The enzymic reaction was not buffer ion-dependent. The optimal pH of oleoyl-12-hydroxylase has not been reported previously although pHs of 7.0 (5,8), 7.1 (3), and 7.2 (7) were used in the incubations with oleoyl-CoA as substrate. Figure 8 shows that inclusion of BSA (1 mg) and CoA (0.5 μmol) does not affect oleoyl-12-hydroxylase while catalase (1000 units) enhances the activity. Although the microsomal fraction used in the incubations was suspended in buffer containing catalase, further addition of catalase to the incubation increased activity by about 10%. ATP (0.5 μmol) and $MgCl_2$ (0.5 μmol) enhance the activity slightly, and the effects may be due to some secondary effect. The cofactor, NADH, could be substituted with NADPH but led to less activity than NADH (Fig. 8), as previously reported (3).

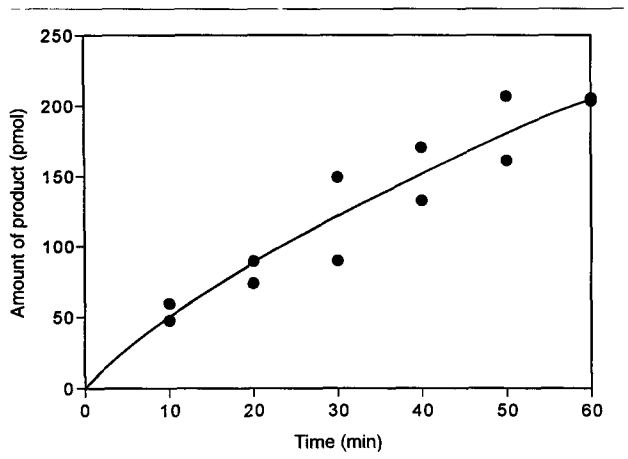


FIG. 6. Ricinoleate produced at various incubation times using microsomal fraction from castor bean.

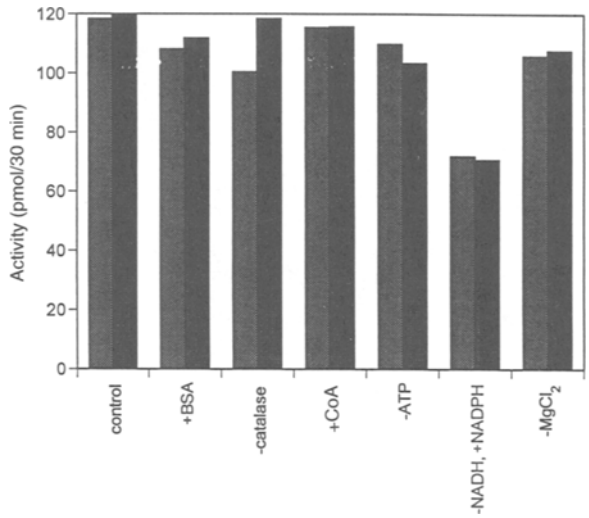
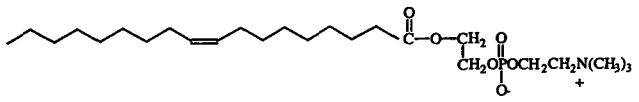


FIG. 8. Effect of some incubation components on the activity of oleoyl-12-hydroxylase in the microsomal fraction from castor bean. For control incubation, see microsomal incubation of the Experimental Procedures section. Two bars represent two determinations; BSA, bovine serum albumin.



SCHEME 1

The PC analogue, OEPC (Scheme 1), strongly inhibits the activity of oleoyl-12-hydroxylase as is shown in Figure 9. An incubation mixture containing 200 μM OEPC produced virtually no ricinoleate, with half inhibition at 40 μM. We do not know the precise mechanism of inhibition. Possible mechanisms are the passive binding to the active site blocking the hydroxylation, hydroxylation of the oleate of OEPC itself, or a detergent effect. To rule out the detergent effect, the inhibition by 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine, an ether lipid structurally similar to OEPC, was studied and the results are shown in Figure 10. Hexadecyl-lysoPC also showed a strong inhibition on oleoyl-12-hydroxylase. We have not yet ruled out any of the proposed mechanisms for OEPC inhibition of the hydroxylase.

Under optimal incubation conditions, 90% of the radioactivity at the end of incubation was associated with PC when the intact lipids were analyzed by TLC. Based on fatty acid methyl ester analysis, this PC fraction included the radioactive substrate and the putative product of oleoyl-12-hydroxylase, 1-acyl-2-ricinoleoyl-*sn*-glycero-3-PC, and a relatively small amount of the likely product of oleoyl-12-desaturase, 1-acyl-2-linoleoyl-*sn*-glycero-3-PC. These three PCs were not separated, and the positional analysis was not done. About 5.5% of the radioactive oleate was converted to ricinoleate. Thus, about 85–90% of the radioactive substrate, 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-PC, still remained at the end of the incubation period. The other radioactive intact lipids shown on TLC plates corresponded to phosphatidic acid, neutral lipids, lysophosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol in the order of decreasing radioactivity.

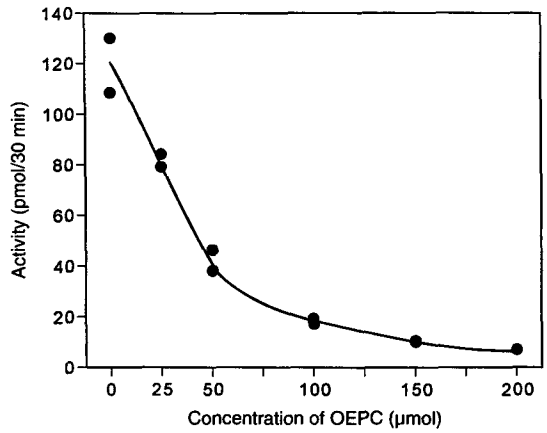


FIG. 9. Effect of oleoyloxyethyl phosphocholine (OEPC) on the activity of oleoyl-12-hydroxylase. Two determinations at each concentration.

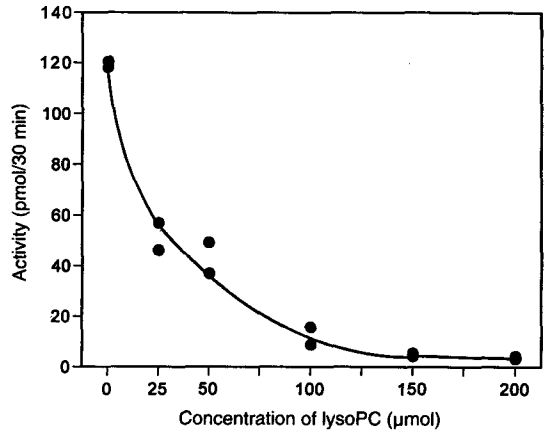


FIG. 10. Effect of 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine on the activity of oleoyl-12-hydroxylase.

The ability to use 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-PC to assay provides us with a means to assay the oleoyl-12-hydroxylase in isolation from endogenous microsomal enzymes required for incorporation of [^{14}C]oleate from oleoyl-CoA into PC. This report represents the first characterization of oleoyl-12-hydroxylase using the endogenous substrate directly.

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Eicosapentaenoic Acid, But Not Docosahexaenoic Acid, Increases Mitochondrial Fatty Acid Oxidation and Upregulates 2,4-Dienoyl-CoA Reductase Gene Expression in Rats

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ABSTRACT: The aim of the present study was to investigate whether eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) was responsible for the triglyceride-lowering effect of fish oil. In rats fed a single dose of EPA as ethyl ester (EPA-EE), the plasma concentration of triglycerides was decreased at 8 h after acute administration. This was accompanied by an increased hepatic fatty acid oxidation and mitochondrial 2,4-dienoyl-CoA reductase activity. The steady-state level of 2,4-dienoyl-CoA reductase mRNA increased in parallel with the enzyme activity. An increased hepatic long-chain acyl-CoA content, but a reduced amount of hepatic malonyl-CoA, was obtained at 8 h after acute EPA-EE treatment. On EPA-EE supplementation, both EPA (20:5n-3) and docosapentaenoic acid (DPA, 22:5n-3) increased in the liver, whereas the hepatic DHA (22:6n-3) concentration was unchanged. On DHA-EE supplementation retroconversion to EPA occurred. No statistically significant differences were found, however, for mitochondrial enzyme activities, malonyl-CoA, long-chain acyl-CoA, plasma lipid levels, and the amount of cellular fatty acids between DHA-EE treated rats and their controls at any time point studied. In cultured rat hepatocytes, the oxidation of [1-^{14}C]palmitic acid was reduced by DHA, whereas it was stimulated by EPA. In the *in vivo* studies, the activities of phosphatidate phosphohydrolase and acetyl-CoA carboxylase were unaffected after acute EPA-EE and DHA-EE administration, but the fatty acyl-CoA oxidase, the rate-limiting enzyme in peroxisomal fatty acid oxidation, was increased after feeding these n-3 fatty acids. The hypocholesterolemic properties of EPA-EE may be due to decreased 3-hydroxy-3-methylglutaryl–CoA reductase activity. Furthermore, replacement of the ordinary fatty acids, i.e., the monoenes (16:1n-7, 18:1n-7, and 18:1n-9) with EPA and some conversion to DPA concomitant with increased fatty acid oxidation is probably the mechanism leading to changed fatty acid composition. In contrast, DHA does not stimulate fatty acid oxidation and, consequently, no such displacement mechanism operates. In conclusion, we have obtained evidence that EPA, and not DHA, is the fatty acid primarily responsible for the triglyceride-lowering effect of fish oil in rats.
Lipids 31, 579–592 (1996).

Administration of marine fish oil rich in n-3 polyunsaturated fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), decreased plasma triglycerides by 25–35% in normolipidemic subjects (1,2) and in rats (3,4). Their greatest effect on plasma triglycerides is under conditions of overproduction when lipogenesis is increased (5,6). There is considerable evidence that increased dietary intakes of n-3 fatty acids reduce hepatic synthesis of very low density lipoproteins (VLDL) triglycerides in humans (5,7,8), as well as in various animal species and in HepG2 cells (9–11). In cultured rat hepatocytes, n-3 fatty acids also have been reported to impair assembly and/or secretion of VLDL (12,13). Moreover, it has been reported that changes in *de novo* fatty acid synthesis is integral to the adaptation to n-3 fatty acids. Short-term experiments with hepatocyte suspensions and primary cultures (14–16) indicate that EPA and DHA are either poor substrates for triglyceride synthesis or that these fatty acids acutely affect the triglyceride synthetic pathway. Inhibition of hepatic VLDL secretion appeared to be secondary to the effects on triglyceride synthesis in those studies. Furthermore, Otto *et al.* (13) reported a positive relationship between fasting plasma insulin and plasma triglycerides and suggested that insulin contributes to the regulation of plasma triglycerides by fish oil.

In addition to the effect on VLDL, many other pathways of hepatic fatty acid metabolism may be affected by fish oil and contribute to the triglyceride-lowering effect. Such mech-

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Abbreviations: ADGAT, acyl-CoA:1.2-diacylglycerol acyltransferase; CMC, carboxymethylcellulose; DHA, docosahexaenoic acid; DHA-EE, docosahexaenoic acid as ethyl ester; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; EPA-EE, eicosapentaenoic acid as ethyl ester; FFA, free fatty acids; GPAT, glycerol-3-phosphate acyltransferase; HMG, hydroxy-3-methylglutaryl; LCFA, long-chain fatty acids; Palm, palmitic acid; PAP, phosphatidate phosphohydrolase; VLCFA, very long-chain fatty acids; VLDL, very low density lipoprotein.

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anisms include suppression of phosphatidate phosphohydrolase (PAP) (17), the enzyme catalyzing the esterification of phosphatidic acid to diglycerides, and acyl-CoA:1.2-diacylglycerolacyltransferase (ADGAT) (14), which mediates the next step. In animals fed high fish oil diets, lowered circulating triglycerides have been associated with increased peroxisomal β-oxidation (3,18) and ketone body formation (19), indicating that increased peroxisomal fatty acid oxidation may be responsible for the hypotriglyceridemic effect. Moreover, Surette *et al.* (20) have shown an association between circulating triglyceride levels and mitochondrial fatty acid oxidation after n-3 polyunsaturated fatty acid feeding. However, it is not known whether these changes in liver enzyme activities and fatty acid oxidation are related to the effect of EPA or DHA or both.

Nearly all studies that have been conducted have used a mixture of the two components of fish oil attracting the most attention, namely EPA and DHA. In our previous reports from feeding experiments in rats administered highly purified EPA and DHA as ethyl esters (EPA-EE and DHA-EE, respectively) for 10–15 d (21,22), EPA, and not DHA, appeared to be responsible for the triglyceride-lowering effect observed. Feeding rats highly purified EPA ethyl ester lowered plasma triglycerides within 1–2 d of treatment, and stimulation of mitochondrial β-oxidation was observed as a primary event (21). We also showed that mitochondrial β-oxidation was stimulated in cultured hepatocytes in the presence of EPA (21). In contrast, administration of highly purified DHA ethyl ester in three animal models produced neither hypotriglyceridemic nor hypocholesterolemic effects, and no effect on mitochondrial β-oxidation was observed, whereas peroxisomal β-oxidation was stimulated after both EPA and DHA feeding (21,22). These results confirm and extend previous findings in normo- and hypertriglyceridemic rats (23,24), perfused rat livers (25), and humans (26), but are in contrast to other reports (27,28).

It is well documented that fish oil feeding decreases plasma cholesterol in rats (29,30), whereas in human studies there is no consistent cholesterol-lowering effect after fish oil feeding (31). The mechanism behind the hypocholesterolemic effect of n-3 fatty acids in rats is not known in detail, but it has been reported that the cholesterol-lowering effect is accompanied by a reduction of the 3-hydroxy-3-methylglutaryl (HMG)–CoA reductase activity (29).

A controversy exists concerning the efficacy of highly concentrated n-3 fatty acids in the form of alkyl esters compared to less concentrated natural triglycerides (32–34). During prolonged feeding, ethyl esters of n-3 fatty acids are highly bioavailable (35,36), whereas the markedly slower hydrolyzation by pancreatic lipase theoretically could influence the absorption of EPA-EE and DHA-EE in short-term studies. In this study we had the opportunity to address the question of whether EPA or DHA as ethyl esters executes the biological effects independent of differences in the absorption.

To precisely determine the biological role of fish oil constituents (n-3 fatty acids), it is imperative that experiments with highly purified n-3 polyunsaturated fatty acids are executed. We agree with Ikeda *et al.* (23) that determining the effects of dietary EPA and DHA on hepatic fatty acid and triglyceride synthesis and β-oxidation seems to be important to clarifying the precise mechanism(s). Therefore we studied the acute effects of a single dose of highly purified EPA-EE and DHA-EE on plasma lipids and on key enzymes in lipid metabolism, focusing on mitochondrial and peroxisomal β-oxidation, to contribute to the understanding of the triglyceride-lowering mechanism of EPA and/or DHA.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Palmitic acid (56 mCi/mmol), L-[U-^{14}C]-glycerol-3-phosphate (171 mCi/mmol), [^{14}C]acetyl-CoA (60 mCi/mmol), and $NaH^{14}CO_3$ (0.1 mCi/mmol) were purchased from the Radiochemical Center (Amersham, England). EPA-EE (purity 95%) and DHA-EE (purity 93%) were obtained from Norsk Hydro AS, Research Center (Porsgrunn, Norway). Palmitic acid (Palm) was from Sigma Chemical Co., (St. Louis, MO). All other chemicals were obtained from common commercial sources and were of reagent grade.

Care of animals. One hundred fifty male Mol:WIST rats from Möllegaard Breeding Laboratory (Ejby, Denmark), weighing approximately 170 g, were housed in pairs in bottom-grid metal wire cages in a room with a controlled temperature (20 ± 3°C), humidity and air changes, and with a 12-h light/dark cycle (light 0700–1900 h). The animals were acclimatized for one week under these conditions before the start of the experiment. All animals had free access to water and food. The rats were fed a conventional pelleted chow diet, RM1 expanded diet (Special Diet Services, Whitam, United Kingdom).

EPA-EE, DHA-EE, and Palm were suspended in 0.5% sodium carboxymethylcellulose (CMC) and 0.5% α-tocopherol. A single dose of 2000 mg/kg body weight was administered by gavage in volumes of 0.8–1.0 mL. In order to provide an equal amount of energy, the control animals received Palm. There were no significant differences in food consumption or weight gain between the experimental groups.

At the time of sacrifice (2, 4, 8, 12, and 24 h after administration), rats were anesthetized with 0.6 mL Hypnorm Dormicum (fluanisone-fentanyl-midazolam) injected subcutaneously in the neck. Anesthesia depth was evaluated by means of the interdigital and palpebral reflexes. The anesthesia was sufficiently deep when there was no response to either of the reflexes (usually within 2–3 min). None of the animals responded to initial abdominal opening. Cardiac puncture was performed, blood was collected into a syringe containing 0.25 mL of 0.2 M EDTA (pH 7.4), and plasma was collected by centrifugation at 1000 × *g* for 10 min at 4°C. The animal experiments were approved by the Norwegian State Board of Biological Experiments with Living Animals.

Preparation of subcellular fractions. The livers were removed, weighed, and parts were immediately chilled on ice and the other parts freeze-clamped in liquid N_2 and stored at

–80°C. Portions of the livers from individual rats were homogenized in ice-cold sucrose-medium (0.25 M sucrose in 10 mM HEPES buffer, pH 7.4, and 1 mM EDTA). Mitochondrial, peroxisomal, microsomal, and cytoplasmic fractions were prepared as described earlier (37). The fractions were stored at –80°C.

Enzyme assays. PAP was determined by measuring the release of free phosphate in the presence of 5 mM $MgCl_2$ (38). Glycerol-3-phosphate acyltransferase (GPAT) was assayed for 3 min at 30°C, as essentially described earlier (39). The reaction was started by the addition of mitochondria or microsomes (200 μg protein), and terminated by the addition of 2.0 mL of 1-*n*-butanol. The incorporation of ^{14}C into butanol-soluble products was determined as described (39,40). ADGAT was assayed for 5 min at 37°C. The reaction was initiated by the addition of microsomes (30–40 μg protein), and terminated by the addition of 7.5 mL of 2-propanol/hexane/water (89:20:16). The extraction of triglycerides into hexane was performed as previously described (41). HMG–CoA reductase activity was measured in the isolated microsomal fraction essentially as previously described (42).

Fatty acyl-CoA oxidase activity (43) and mitochondrial β-oxidation, using radiolabeled palmitoyl-L-carnitine and L-carnitine in the presence of palmitoyl-CoA (43), were determined as described earlier. Acetyl-CoA carboxylase activity was measured in the cytosolic fraction as fixation of $^{14}CO_2$ from $NaH^{14}CO_3$ into malonyl-CoA essentially as described earlier (44). 2,4-Dienoyl-CoA reductase activity was measured in the mitochondrial fraction, and the incubation contained 50 mM KH_2PO_4/K_2HPO_4 pH 7.4, 0.01% (vol/vol) Triton X-100 (Sigma Chemical Co.), 100 μM NADPH, and 100 μg protein. The assay was started by the addition of 100 μM 2-*trans*, 4-*cis*-decadienoyl-CoA and run for 10 min at 30°C at 340 nm (45).

Determination of malonyl-CoA and long-chain acyl-CoA. Malonyl-CoA and free CoASH levels were quantitated by a modification of the high-performance liquid chromatography method originally described by Corkey *et al.* and Demoz *et al.* (46,47). Briefly, 0.1 g liver was homogenized in 1 mL 5% sulfosalicylic acid containing 50 mM dithioerythritiol. The sample was centrifuged at 600 × *g* for 10 min at 0°C. Twenty μL of the acid-soluble extracts were injected into a Spectra Physics (San Jose, CA) SP 8800 HPLC system. Absorbance measurements were made at 254 nm using a 3 mm Hypersil C18 reverse-phase column (HiChrom Ltd., Reading, Berkshire, England). The mobile phase was 0.1 M NaH_2PO_4, pH 4.9 (buffer A) and buffer B (a mixture of buffer A and methanol in a ratio of 7:3), pH 4.9. The flow rate was 2.0 mL/min. The profile of the elution was as follows: 0–10 min: 10–40% B; 10.1–17.6 min: 40–90% B. The retention times were 9.3 and 13.7 for free CoA and malonyl-CoA, respectively. Total hepatic long-chain acyl-CoA was estimated by incubating liver tissue precipitates with 1.5 M KOH at 55°C for 1 h. The liberated free CoASH found in the supernatant was determined as described previously. Individual working standards were prepared by dissolving malonyl-CoA or free CoASH, for determination of malonyl-CoA or long-chain acyl-CoA, respectively, in 5% sulfosalicylic acid containing 50 μM of dithioerythritol. They were then mixed to give a final concentration of 100 μM. Standard curves of the different CoA-esters were obtained at 25, 50, 100, and 200 mM concentrations and showed linear conditions ($r^2 > 0.999$; Ref. 47). The recovery obtained by the method was approximately 90%.

Lipid analysis. Protein was assayed by a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA). Lipid analyses were carried out by the Monotest cholesterol enzymatic kit (Boehringer, Mannheim, Germany), the Monotest phospholipid enzymatic kit (Boehringer), and the Biopak Triglyceride enzymatic kit (Biotrol, Paris, France). Hepatic lipids were quantified after extraction from total liver homogenates (48). Plasma free fatty acids were determined by an enzymatic colorimetric method (WACO NefaC; Waco Chemicals, Neuss, Germany) (49,50).

Total fatty acid determination. Total lipids were extracted from liver as described by Lie *et al.* (51). The lipid fractions were evaporated, saponified, 19:0 was added as internal standard, and the fatty acids esterified in 12% BF_3 in methanol. The methyl esters were separated using a Carlo Erba (Milan, Italy) 2900 gas-chromatograph ("cold on column" injection, starting at 60 and increasing at 49°C/min to 160, increasing at 1°C/min to 190, increasing at 4°C/min to 220°C), equipped with a 50 m CP-Sil 88 (Chrompack) fused silica capillary column (0.32-mm i.d.). The fatty acid composition was calculated using a Maxima 820 Chromatography Workstation software, installed in an IBM-AT, connected to the gas–liquid chromatograph and identification ascertained by standard mixtures of methyl esters (Nu-Chek-Prep, Elysian, MN). Certified reference material (CRM 162, CRM 163) from Community Bureau of Reference (BCR, Brussels, Belgium) was used.

Preparation of cultured hepatocytes. Hepatocytes were prepared as described earlier (52). In short, the rats were anesthetized as described here, and hepatocytes were isolated by collagenase perfusion, washed and plated in Dulbecco's modified Eagle's medium containing HEPES (20 mM), Ultroser G (2%), and gentamicin (50 mL) at a density of 3×10^6 cells/dish or culture flask (1×10^6 cells/mL). The culture medium was replaced after 6–7 h (2 mL/dish, medium as before). The cells were incubated at 37°C in an atmosphere of air containing 5% CO_2.

Fatty acid oxidation. Fatty acid oxidation to acid-soluble products and CO_2 (to determine the rate of β-oxidation) was measured as described earlier (53). Two mL Dulbecco's modified Eagle's medium containing HEPES (20 mM), gentamicin [1-^{14}C] Palm (0.5 μCi/mL, 200 μM), and 500 μM L-carnitine hydrochloride was added to the cultured hepatocytes, and the hepatocytes were incubated for 4 and 9 h in the presence of increasing concentrations of EPA and DHA (see Fig. 1).

The culture flasks contained a center well (Kontes, Vineland, NJ) and a folded filter paper and were closed with stopper tops (Kontes). After incubation, 300 μL of phenylethylamine/methanol (1:1, vol/vol) was added to the center

well and 300 mL of $HClO_4$ (1 M) to the cell through the stopper top by means of a syringe, and the flasks were further incubated for 1 h at room temperature to trap all $^{14}CO_2$. After incubation, the well was cut off, and radioactivity was measured by liquid scintillation counting.

Preparation of hybridization probes. DNA fragments were labelled by random priming using the oligolabelling technique of Feinberg and Vogelstein (54), resulting in specific activities ranging from 0.8 to 5 × 10^9 cpm/mg DNA. The DNA probes were purified fragments of cloned rat genes: (i) 2,4-dienoyl-CoA reductase: 632 bp Pst I/Eco RI insert in pGem-4Z and (ii) 28 S rRNA: 1.4 kb Bam HI fragment of pA (Dr. I.L. Gonzales, personal communication).

RNA purification and analysis. Total cellular RNA was isolated by the guanidinium-thiocyanate method described by Chomczynski and Sacchi (55). The RNA concentrations were determined spectrophotometrically. Slot blotting of RNA onto nylon was carried out as described by Aasland *et al.* (56). Hybridization to immobilized RNA was performed as described by Sambrook *et al.* (57), in the presence of 50% formamide, 5 × saline–sodium–citrate, 200 μg/mL heat denatured herring-sperm DNA, 0.1% SDS, 25 mM sodium phosphate pH 6.5, 8.25% dextran sulfate at 42°C for 24 to 48 h. Filters were washed to high stringency (0.2 × saline–sodium–citrate, 0.1% NaPPi, 0.1% SDS at 65°C) and exposed on Kodak XAR-5 X-ray films in the presence of intensifying screens at −80°C. Densitometric scanning of autoradiograms was performed using the LKB Ultrogel laser-densitometer (Bromma, Sweden), or the hybridized membranes were analyzed by Instant Imager, Electronic Audioradiography (Packard, Downer's Grove, IL). When the filters were to be rehybridized, the bound probe was first stripped off in 0.1% SDS at 90–100°C for 7 min. The hybridization results were normalized to the signal of 28 S rRNA hybridization in the individual samples. Relative mRNA induction of the different genes was then calculated.

Statistical analysis. The *in vivo* results are means ± SD of duplicate or triplicate measurements on six animals of each experimental group. Results from cultured rat hepatocytes are presented as means ± SD of at least three culture dishes/flasks of hepatocytes from two or more animals.

The effects of EPA and DHA treatment compared to controls were tested by a one-way analysis of variance. Fisher's Least Square Difference was used to determine the significance of difference between the mean at 95% confidence interval using a statistical software StatView SE + Graphics on an Apple Macintosh. $P < 0.05$ was taken to be statistically significant.

RESULTS

Effect of n-3 fatty acids on oxidation of [1-^{14}C]Palm in cultured rat hepatocytes. In agreement with earlier findings (21), oxidation of [1-^{14}C]Palm was significantly higher in cells grown with L-carnitine than in cells grown without L-carnitine (data not shown). Oxidation of Palm to carbon dioxide and acid-soluble products was significantly stimulated in cultured hepatocytes in the presence of EPA, whereas it was significantly reduced in the presence of DHA (Fig. 1A). Oleic acid had no effect on Palm oxidation (data not shown). A significant difference in Palm oxidation by EPA and DHA was already observed after 4 h of incubation (Fig. 1B).

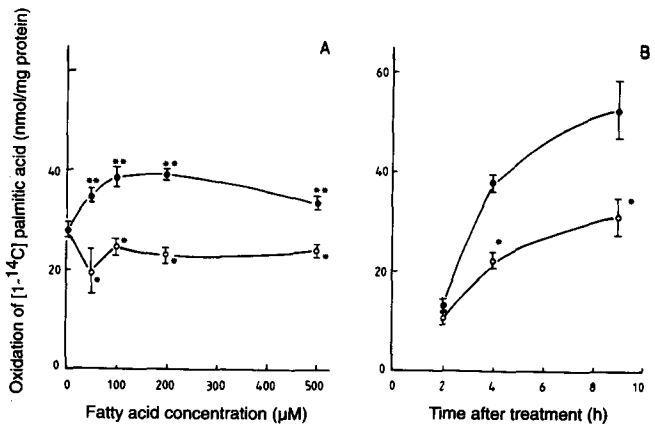


FIG. 1. Effect of eicosapentaenoic acid (EPA) (●) and docosahexaenoic acid (DHA) (○) on oxidation of [1-^{14}C]palmitic acid (0.2 mM) in cells plated and incubated with increasing concentrations (A) and for different times (B). A, cultured hepatocytes were incubated for 4 h; B, 200 μM EPA and DHA was used. In all experiments the fatty acid/bovine serum albumin ratio was 2.5:1, and 0.5 mM L-carnitine was added to the medium. Acid-soluble radioactivity was determined as described in the Materials and Methods section. Results are given as means ± SD for values obtained from three independent experiments. Asterisks correspond to the statistical comparison between values from EPA and DHA and values from DHA and controls (*) and values from EPA and controls (**).

Acute treatment. As shown in Table 1 plasma triglycerides, cholesterol and phospholipids were not changed at any of the time points studied in the different control groups. As shown in Figure 2, plasma triglycerides and cholesterol were reduced 8 h after the acute treatment with EPA-EE as compared to controls. Restoration to control levels occurred at 12 and 24 h. Plasma triglycerides and cholesterol were unaffected by DHA-EE treatment (Fig. 2). Figure 3A shows that plasma phospholipids were reduced 8 h after acute treatment with EPA-EE as compared to controls and DHA-EE administration. The plasma free fatty acid (FFA) concentration in the control and DHA-EE treated rats was significantly reduced at 4 h compared to 2 h, and this reduced level was maintained up to 12 h after feeding. The FFA concentration in the EPA-EE treated rats maintained high until 8 h time point, when it was reduced to the same level as the other two groups (Fig. 3B). No statistically significant difference was found either for hepatic triglycerides, cholesterol, phospholipids (Table 2), or for activities of PAP, acetyl-CoA carboxylase (Table 2), and GPAT (data not shown) between EPA-EE and DHA-EE treated rats and their controls at any time of the time points studied, except that PAP was reduced 8 and 12 h after acute treatment with DHA-EE as compared to controls. However, EPA-EE and DHA-EE feeding caused increased fatty acyl-CoA oxidase activity at 12 h compared to controls (Table 3).

TABLE 1
Plasma Lipids for Three Control Groups, Receiving Either Pellet Diet, Carboxymethylcellulose, or Palmitic Acid[a]

Number of animals	Time (h)	Palmitic acid			Carboxymethylcellulose			Pellet diet		
		TG	CHOL	PL	TG	CHOL	PL	TG	CHOL	PL
6	2	1.18 ± 0.57	1.45 ± 0.08	1.64 ± 0.16	1.37 ± 0.45	1.60 ± 0.31	1.66 ± 0.10	1.27 ± 0.29	1.42 ± 0.27	1.69 ± 0.12
6	4	1.16 ± 0.61	1.59 ± 0.21	1.78 ± 0.16	1.37 ± 0.60	1.45 ± 0.29	1.67 ± 0.06	1.59 ± 0.30	1.31 ± 0.28	1.59 ± 0.27
6	8	1.54 ± 0.40	1.53 ± 0.13	1.68 ± 0.15	1.10 ± 0.57	1.59 ± 0.19	1.65 ± 0.24	1.46 ± 0.04	1.54 ± 0.04	1.86 ± 0.07
6	12	1.41 ± 0.45	1.72 ± 0.28	1.85 ± 0.21	0.96 ± 0.15	1.64 ± 0.22	1.75 ± 0.11	1.37 ± 0.41	1.62 ± 0.36	1.90 ± 0.36
6	24	1.62 ± 0.25	1.61 ± 0.09	1.81 ± 0.09	1.55 ± 0.36	1.56 ± 0.21	1.68 ± 0.07	1.03 ± 0.23	1.73 ± 0.03	1.69 ± 0.17

[a]TG, triglycerides; CHOL, cholesterol; PL, phospholipids. Plasma concentrations of lipids are given in nmol/L and were analyzed as described in the Materials and Methods section. The values are expressed as means ± SD. There were no statistical differences between the groups evaluated by one-way analysis of variance and Fisher's Protected Least Square Differences used to determine differences between means at 95% confidence interval.

As shown in Table 3, the 2,4-dienoyl-CoA reductase activity was not changed at any of the time points studied in the control group, and no statistically significant difference was found for the activity between DHA-EE treated rats and their controls. As also shown in Table 3, the reductase activity was increased 4 and 12 h after acute treatment with EPA-EE as compared to controls and DHA-EE administration.

Figure 4A summarizes the activities of fatty acid oxidation in mitochondrial fraction in treated rats and controls at different times after acute treatment. Oxidation of fatty acids in the mitochondrial fraction, using palmitoyl-L-carnitine as substrate, was significantly increased 8 h after acute treatment with EPA-EE as compared to controls, and this difference disappeared at 12 and 24 h (Fig. 4A). The oxidation of fatty acids in mitochondrial fraction was decreased 12 and 24 h after acute treatment with DHA-EE as compared to controls

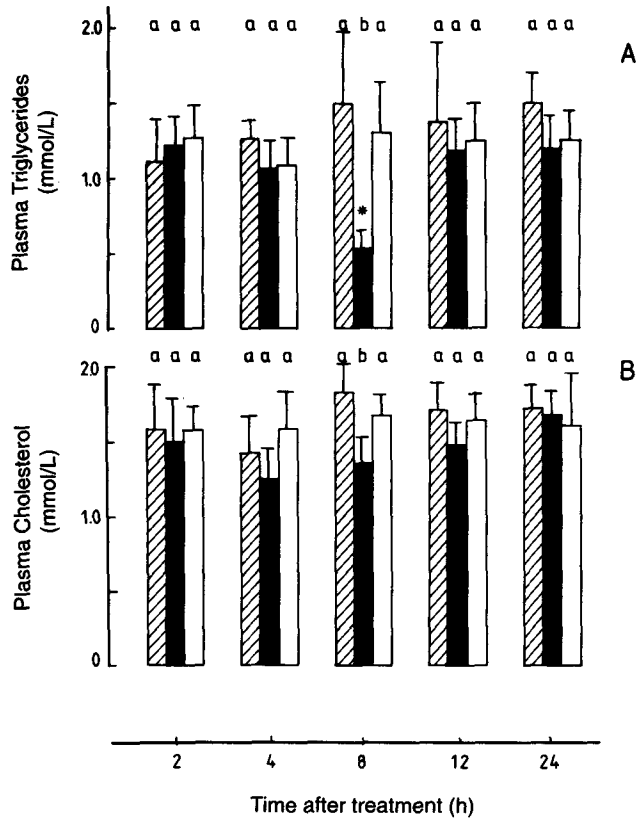


FIG. 2. Concentrations of plasma triglycerides (A) and cholesterol (B) at different times after a single oral administration of EPA as ethyl esters (EPA-EE) (black columns) and DHA as ethyl esters (DHA-EE) (open columns) in the rat and of controls (hatched columns). The results are presented as means ± SD of six rats per group. *Significantly different from each of the time points studied ($P < 0.05$). Means in a column with different superscripts are significantly different ($P < 0.05$) determined by one-way analysis of variance, Fisher's test. Abbreviations as in Figure 1.

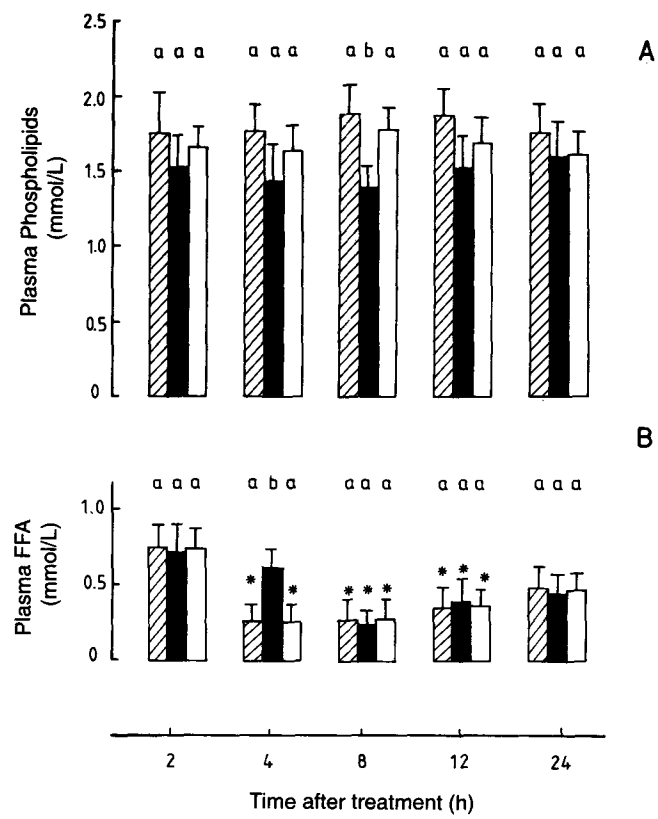


FIG. 3. Concentrations of plasma phospholipids (A) and free fatty acids (FFA) (B) at different times after a single oral administration of EPA-EE (black columns) and DHA-EE (open columns) in the rat and of controls (hatched columns). The results are presented as means ± SD of six rats per group. *Significantly different from values at two hours of individual treatment ($P < 0.05$). Means in a column with different superscripts are significantly different ($P < 0.05$) determined by one-way analysis of variance, Fisher's test. Abbreviations as in Figure 2.

TABLE 2
Liver Lipids and Activities of Phosphatidate Phosphohydrolase (PAP) and Acetyl-CoA Carboxylase at Different Times After Acute EPA-EE and DHA-EE Administration in the Rat[a]

	Hours	EPA-EE	*P*	Control	*P*	DHA-EE
Triglycerides	2	3.54 ± 0.62[a]	n.s.	3.17 ± 0.52[a]	n.s	2.89 ± 0.70[a]
(nmol/g liver)	4	3.34 ± 0.81[a]	n.s.	3.14 ± 0.63[a]	n.s.	3.06 ± 0.64[a]
	8	3.18 ± 0.56[a]	n.s.	2.90 ± 0.63[a]	n.s.	3.59 ± 0.36[a]
	12	2.86 ± 0.74[a]	n.s.	2.81 ± 0.46[a]	n.s.	3.84 ± 0.52[a]
	24	2.89 ± 0.69[a]	n.s.	3.19 ± 0.84[a]	n.s.	4.03 ± 0.93[a]
Cholesterol	2	1.40 ± 0.21[a]	n.s.	1.46 ± 0.21[a]	n.s.	1.18 ± 0.34[a]
(nmol/g liver)	4	1.22 ± 0.32[a]	n.s.	1.36 ± 0.12[a]	n.s.	1.04 ± 0.26[a]
	8	1.65 ± 0.32[a]	n.s.	1.45 ± 0.22[a]	n.s.	1.53 ± 0.42[a]
	12	1.95 ± 0.36[a]	n.s.	1.94 ± 0.21[a]	n.s.	1.78 ± 0.41[a]
	24	1.89 ± 0.21[a]	n.s.	1.92 ± 0.34[a]	n.s.	1.75 ± 0.42[a]
Phospholipids	2	12.66 ± 1.60[a]	n.s.	11.21 ± 0.57[a]	n.s.	10.99 ± 1.10[a]
(nmol/g liver)	4	10.71 ± 0.68[a]	n.s.	10.64 ± 1.80[a]	n.s.	10.26 ± 0.58[a]
	8	11.87 ± 0.93[a]	n.s.	11.48 ± 1.10[a]	n.s.	10.86 ± 1.70[a]
	12	9.54 ± 1.90[a]	n.s.	9.52 ± 1.60[a]	n.s.	11.10 ± 0.46[a]
	24	9.48 ± 2.10[a]	n.s.	9.95 ± 1.68[a]	n.s.	10.18 ± 0.63[a]
PAP	2	11.8 ± 1.2[a]	n.s.	12.8 ± 1.7[a]	n.s.	14.2 ± 1.8[a]
activity	4	12.4 ± 1.1[a]	n.s.	11.9 ± 2.0[a]	n.s.	11.5 ± 0.7[a]
(nmol/min/mg	8	11.5 ± 1.4[a]	n.s.	11.8 ± 0.6	<0.05	9.5 ± 1.2[b]
protein)	12	10.0 ± 1.3[a]	n.s.	11.3 ± 0.6[a]	<0.05	7.9 ± 0.5[b]
	24	9.2 ± 1.6[a]	n.s.	10.3 ± 1.3[a]	n.s.	9.4 ± 0.9[b]
Acetyl-CoA	2	21.4 ± 2.5[a]	n.s.	24.3 ± 2.6[a]	n.s.	28.4 ± 2.5[a]
carboxylase	4	27.3 ± 2.6[a]	n.s.	28.4 ± 1.1[a]	n.s.	23.8 ± 5.2[a]
activity	8	22.6 ± 2.1[a]	n.s.	28.7 ± 3.4[a]	n.s.	27.6 ± 2.7[a]
(nmol/min/mg	12	25.8 ± 2.3[a]	n.s.	26.9 ± 4.6[a]	n.s.	30.4 ± 2.3[a]
protein)	24	20.9 ± 1.6[a]	n.s.	26.9 ± 3.1[a]	n.s.	25.1 ± 5.1[a]

[a]PAP activity was measured in the isolated microsomal fraction, whereas the acetyl-CoA carboxylase was determined in the cytosol. Statistical comparison between eicosapentaenoic acid as ethyl ester (EPA-EE), docosahexaenoic acid as ethyl ester (DHA-EE), and controls are shown by the *P* values; n.s., not significant. [a,b]Correspond to the statistical comparison between the groups at each of the time points studied (hours), where the same letter within the same parameter means that there is no statistical difference between the corresponding groups evaluated by one-way analysis of variance and Fisher's Protected Least Square Difference used to determine differences between means at 95% confidence interval.

(Fig. 4A). Thus, statistically significant difference was found for mitochondrial β-oxidation between the EPA-EE and DHA-EE treated rats from 8 to 24 h after receiving the diets, using palmitoyl-L-carnitine (Fig. 4A) and palmitoyl-CoA as substrates (data not shown). When rats were treated with EPA-EE, the 2,4-dienoyl-CoA reductase mRNA concentration in-

TABLE 3
Fatty Acyl-CoA Oxidase Activity and 2,4-Dienoyl-CoA Reductase Activity at Different Times After Acute EPA-EE and DHA-EE Administration in the Rat[a]

	Hours	EPA-EE	*P*	Controls	*P*	DHA-EE
Fatty acyl-CoA	2	13.3 ± 2.4[a]	n.s.	15.0 ± 3.6[a]	n.s.	19.0 ± 1.4[a]
oxidase activity	4	17.9 ± 1.6[a]	n.s.	14.6 ± 3.0[a]	n.s.	17.7 ± 2.9[a]
(nmol/min/mg	8	17.7 ± 2.3[a]	n.s.	13.6 ± 2.0[a]	n.s.	16.6 ± 2.4[a]
protein)	12	19.4 ± 2.1[b]	<0.05	14.7 ± 2.3[a]	<0.05	19.1 ± 1.3[a]
	24	16.8 ± 1.8[a]	n.s.	13.1 ± 2.6[a]	n.s.	15.4 ± 2.7[a]
2,4-Dienoyl-CoA	2	1.28 ± 0.18[a]	n.s.	1.30 ± 0.16[a]	n.s.	1.65 ± 0.24[a]
reductase activity	4	1.91 ± 0.31[a]	<0.05	1.23 ± 0.28[a]	n.s.	1.26 ± 0.36[a,b]
(nmol/min/mg	8	2.02 ± 0.26[b]	<0.05	1.07 ± 0.33[a]	n.s.	0.88 ± 0.14[b]
protein)	12	1.65 ± 0.10[b]	<0.05	0.94 ± 0.17[a]	<0.05	0.94 ± 0.18[b]
	24	1.18 ± 0.07[a]	n.s.	1.05 ± 0.14[a]	n.s.	1.17 ± 0.15[b]

[a]The acyl-CoA oxidase activity was determined in the isolated peroxisome-enriched fraction, whereas the 2.4-dienoyl-CoA reductase activity was measured in the isolated mitochondrial fraction. The values are expressed as means ± SD of six rats per group. Statistical comparison between EPA-EE, DHA-EE, and controls are shown by the *P* values. [a,b]Correspond to the statistical comparison between the groups at each of the time points studied (hours), where the same letter within one enzyme activity means that there is no statistical differences between the corresponding groups ($P < 0.05$). Abbreviations as in Table 2.

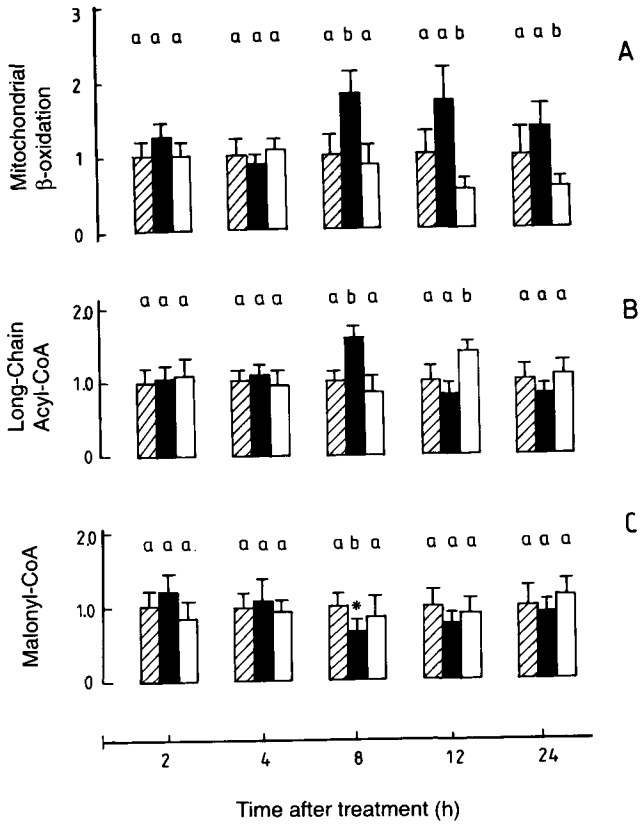


FIG. 4. Relative specific activities of mitochondrial β-oxidation, with palmitoyl-L-carnitine as substrate (A), relative concentration of hepatic long-chain acyl-CoA (B), and relative concentration of liver malonyl-CoA (C) at different times after a single oral administration of EPA-EE (black columns) and DHA-EE (open columns) in the rat. The relative values are calculated to those of palmitic acid-fed controls (hatched columns) = 1.0 at each of the time points studied. The results are presented as means of six rats per group. Means in the column with different superscripts are significantly different ($P < 0.05$) determined by one-way analysis of variance, Fisher's test. *Significantly different from values at two hours of individual treatment ($P < 0.05$). Abbreviations as in Figure 2.

creased significantly 4 h after treatment (Fig. 5), and continued to rise more than 1.5-fold above control values at 12 h after exposure. No evidence was found for induction of 2,4-dienoyl-CoA reductase after acute administration of DHA-EE during this experiment compared to the 28 S rRNA (Fig. 5).

As shown in Figure 4B, hepatic long-chain acyl-CoA content was increased 8 h after acute treatment with EPA-EE as compared to controls and DHA-EE treated rats. At 12 h the long-chain acyl-CoA level in EPA-EE treated rats was normalized. In contrast to the changes of long-chain acyl-CoA level observed after EPA-EE feeding, the malonyl-CoA level was reduced from the second to the eighth hour (Fig. 4C), where the mitochondrial β-oxidation was increased (Fig. 4A), and at 8 h the malonyl-CoA values became significantly lower in EPA-EE treated rats than in controls (Fig. 4C).

As shown in Table 4, the total amount of the different fatty acids in the liver was not changed at any of the time points studied in the Palm treated group (controls). Treating the rats with Palm did not change the fatty acid composition, including the amount of Palm (16:0) compared to rats only treated with CMC (data not shown). The total amounts of monoenes and polyenes were decreased and increased, respectively, 4 h after acute treatment with EPA-EE as compared to Palm (controls) and DHA-EE administration (Table 4). Palmitoleic acid (16:1n-7) and vaccenic acid (18:1n-7) were significantly reduced after EPA-EE treatment compared to DHA-EE feeding, whereas the amount of oleic acid (18:1n-9) was reduced compared to controls. No statistically significant difference was found for either stearic acid (18:0), linoleic acid (18:2n-6), α-linolenic acid (18:3n-3), and arachidonic acid (20:4n-6) between EPA-EE, DHA-EE, and Palm treated rats at any of the time points studied. The total amount of cellular EPA (20:5n-3) and docosapentaenoic acid (DPA) (22:6n-3) increased from the second to the fourth hour after EPA-EE administration, and these fatty acids were increased at all time points studied with EPA-EE compared to controls and DHA-EE feeding. Table 4 further shows that the amount of cellular DHA (22:6n-3) increased at all time points studied in DHA-EE treated rats compared to controls except at 8 h, but 22:6n-3 did not reach a peak at 4 h as 20:5n-3 after EPA-EE administration. Moreover, at all time points studied, the fold increase of 22:6n-3 after DHA-EE treatment was much lower than the increase of 20:5n-3 after EPA-EE feeding. No statistically significant difference was found for 22:5n-3 between controls and DHA-EE administration at any of the time points studied (Table 4). Noteworthy, however, was the fact that DHA-EE supplementation elevated the amount of 20:5n-3 the first 8 h after administration.

As shown in Table 5, the HMG–CoA reductase activity in the control group was increased at 8 h and this increase was maintained to 12 h after feeding. As also shown in Table 5, the HMG–CoA reductase activity was reduced 8 h after acute treatment with EPA-EE as compared to controls and DHA-EE administration.

DISCUSSION

It has been assumed that EPA or DHA, the major polyunsaturated fatty acids of fish oil, or both are responsible for the triglyceride-lowering effect. Most studies have used fish oil with various contents of EPA and DHA, and only a few studies have examined the effect of EPA and DHA separately to determine the effects of each fatty acid. The present study reports that EPA, and not DHA, is the fatty acid primarily responsible for the triglyceride-lowering effect of fish oil in rats. These acute effects are consistent with observations in normolipidemic and hyperlipidemic rats (21–24). Studies in perfused livers and cultured cells have shown that EPA reduces triglyceride synthesis/secretion in perfused rat liver (19,58), in rat (12,59), rabbit hepatocytes (12,59,60), and in HepG2 cells (9,61). Furthermore, Zhang *et al.* (58) observed in a liver perfusion study that only EPA, and not DHA, decreased triglyceride synthesis and VLDL secretion. In contrast, Williams *et al.* (62) observed that feeding pure DHA re-

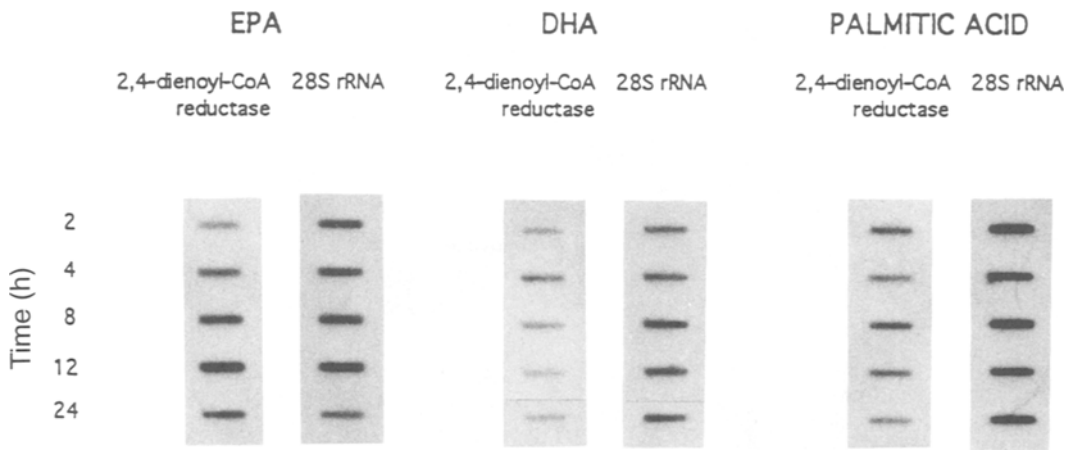


FIG. 5. Kinetics of 2,4-dienoyl-CoA reductase and 28S rRNA after acute treatment with DHA-EE and palmitic acid. One representative slot blot of a total of three from six rats in each group is shown. Mouse 28S rRNA was used as a probe to evaluate the amount of RNA applied on the filter, which was 5, 2, and 1 μg. Abbreviations as in Figure 1.

duced hepatic triglyceride secretion *in vivo*. Similarly, Martin *et al.* (63) reported a reduction of triglyceride secretion in rat hepatocytes following incubation with DHA, but concluded that inhibition of hepatocyte triglyceride synthesis is not obligatory for the 22:6, n-3-induced diminution of triglyceride secretion.

In humans, supplementation with purified EPA has lowered triglycerides in plasma (64–67), whereas the few studies reported on purified DHA on human serum lipoprotein levels are conflicting (26–28,68). Worne and Smith (68) reported a marked reduction in total serum lipids by EPA, by DHA, and by arachidonic acid administration (triglyceride levels were not measured), and Hirai *et al.* (27) showed a significant reduction of serum triglycerides and cholesterol in hypertriglyceridemic patients of Type IIa and Type IIb hyperlipidemia following both highly purified EPA and DHA ingestion. In contrast, a report by Harris (26) at the recent ISSFAL meeting found that EPA, but not DHA, appeared to be responsible for the triglyceride-lowering effect of fish oil in humans. This is in contrast to the Tromsø study, reported at the same meeting, where it was concluded that DHA was more efficient than EPA in decreasing plasma triglyceride concentrations (28). The cause of the differences between the results in the human studies is not clear, but one explanation might be that the Japanese and Norwegian people consume more fish and thereby have higher basal values of EPA and DHA compared to the healthy subjects in Kansas City, Missouri, where fish consumption is lower.

A large number of reports have been published demonstrating that ethyl esters of n-3 fatty acids execute the same biological effects as other chemical forms of these fatty acids (67,69). However, it is clear that the *in vitro* hydrolysis of ethyl esters of very long-chain n-3 fatty acids is markedly reduced as compared to the free forms and triglycerides of these fatty acids (34,70). During prolonged feeding, ethyl esters of n-3 fatty acids are highly bioavailable, whereas the markedly slower hydrolyzation by pancreatic lipase theoretically could influence the absorption of n-3 fatty acids in short-term studies. Our results from fatty acid composition in liver of rats treated with a single dose of EPA-EE and DHA-EE show that EPA-EE feeding increases (20:5n-3) after two hours and that feeding EPA-EE also increases DPA (22:5n-3), whereas DHA (22:6n-3) remains unchanged (Table 4). Furthermore, we observed triglyceride-lowering effect by EPA-EE after eight hours (Fig. 2). Concerning DHA-EE feeding, an increase in fatty acid composition in liver was observed after two hours. Our results show that EPA-EE and DHA-EE are rapidly absorbed and exert their effects on plasma lipids and key enzyme activities of lipid metabolism within hours. Thus, the discrepancy regarding the separate effects of EPA and DHA is not due to the bioavailability of ethyl esters of n-3 fatty acids compared to n-3 fatty acids esterified to triacylglycerol or phosphoacylglycerol.

We had the opportunity in this study to address the question of the mechanism behind this lipid-lowering effect. EPA-EE treatment for either hours (Figs. 2 and 3) or given over a 15-d period (21) to normolipidemic rats decreased plasma triglycerides, cholesterol, and phospholipids. Prolonged treatment with EPA-EE is reported to increase the activity of acyl-CoA:diacylglycerol acyltransferase and to decrease the acetyl-CoA carboxylase activity (21). No change of the last enzyme activity was observed after acute treatment (Table 2). EPA-EE increased mitochondrial fatty acid oxidation (Fig. 4A) and, most plausible, also peroxisomal β-oxidation, as the fatty acyl-CoA oxidase activity was increased (Table 3). EPA-EE treatment either for prolonged feeding (21) or given acutely to rats had no effect on the PAP activity (Table 2). It is conceivable that, whereas the hypolipidemic effect of EPA-EE after prolonged treatment (18,21) may be influenced by alterations of the triglyceride biosynthesis and lipogenesis, its effect after acute treatment appears to be independent of changes of these pathways. The triglyceride-lowering effect after acute EPA-EE treatment may be a consequence of its effect on hepatic fatty acid oxidation.

TABLE 4
Changes in Fatty Acid Composition in Liver of Rats Treated with EPA-EE, DHA-EE, and Palmitic Acid (Palm) (controls)

Fatty acid	Treatment/hours	Fatty acid composition (mol%) 2	4	8	24
16:0	Palm	20.6 ± 1.2[a]	22.2 ± 1.3[a]	20.5 ± 1.1[a]	23.5 ± 2.0[a]
	EPA-EE	19.8 ± 0.7[a]	18.9 ± 1.1[a]	19.6 ± 1.5[a,c]	23.4 ± 0.9[a,c]
	DHA-EE	21.3 ± 0.3[a]	21.3 ± 1.4[a]	23.3 ± 0.9[a]	20.3 ± 1.6[a]
16:1n-7	Palm	2.4 ± 0.5[a]	2.7 ± 0.8[a]	1.9 ± 0.4[a]	3.1 ± 1.6[a]
	EPA-EE	2.2 ± 0.1[a]	2.0 ± 0.2[a,c]	1.6 ± 0.3[b,c]	1.7 ± 0.1[b]
	DHA-EE	2.5 ± 0.1[a]	2.7 ± 0.4[a]	2.8 ± 0.4[a]	2.0 ± 0.4[a]
18:0	Palm	16.8 ± 1.3[a]	17.5 ± 1.6[a]	17.6 ± 1.5[a]	15.1 ± 3.0[a]
	EPA-EE	16.5 ± 0.9[a]	17.1 ± 0.5[a]	16.9 ± 1.0[a]	16.8 ± 0.4[a]
	DHA-EE	16.0 ± 0.5[a]	15.2 ± 1.2[a]	16.5 ± 0.4[a]	16.3 ± 0.6[a]
18:1n-7	Palm	3.3 ± 0.2[a]	3.7 ± 0.6[a]	4.0 ± 1.0[a]	4.1 ± 1.1[a]
	EPA-EE	3.0 ± 0.3[a,c]	3.2 ± 0.3[a,c]	3.7 ± 0.4[a]	3.3 ± 0.3[a]
	DHA-EE	4.0 ± 0.3[a]	4.7 ± 0.5[a]	4.0 ± 0.3[a]	4.2 ± 0.6[a]
18:1n-9	Palm	7.0 ± 1.4[a]	7.8 ± 0.6[a]	5.8 ± 0.2[a]	7.8 ± 2.4[a]
	EPA-EE	6.5 ± 1.1[a]	5.6 ± 0.4[a,d]	5.3 ± 0.4[a]	6.5 ± 0.8[a]
	DHA-EE	5.7 ± 0.5[a]	6.5 ± 0.7[a]	6.2 ± 0.7[a]	5.1 ± 0.4[a]
18:2n-6	Palm	14.3 ± 1.1[a]	13.2 ± 1.3[a]	14.4 ± 1.0[a]	14.8 ± 1.1[a]
	EPA-EE	13.8 ± 0.5[a]	12.5 ± 6.6[a]	13.2 ± 1.0[a]	14.0 ± 1.2[a]
	DHA-EE	13.8 ± 0.4[a]	13.4 ± 1.0[a]	13.3 ± 0.6[a]	14.9 ± 1.2[a]
18:3n-3	Palm	0.2 ± 0.1[a]	0.1 ± 0.0[a]	0.2 ± 0.0[a]	0.2 ± 0.0[a]
	EPA-EE	0.2 ± 0.1[a]	0.2 ± 0.1[a]	0.2 ± 0.0[a]	0.2 ± 0.1[a]
	DHA-EE	0.2 ± 0.0[a]	0.2 ± 0.1[a]	0.2 ± 0.0[a]	0.3 ± 0.0[a]
20:4n-6	Palm	18.0 ± 2.9[a]	16.8 ± 0.8[a]	19.2 ± 1.4[a]	15.7 ± 2.3[a]
	EPA-EE	16.4 ± 1.3[a]	16.7 ± 1.5[a]	18.3 ± 2.2[a]	16.1 ± 0.8[a]
	DHA-EE	16.8 ± 0.5[a]	15.0 ± 1.2[a]	15.8 ± 1.6[a]	18.0 ± 1.8[a]
20:5n-3	Palm	1.1 ± 0.4[a]	0.9 ± 0.2[a]	0.9 ± 0.1[a]	1.0 ± 0.1[a]
	EPA-EE	4.2 ± 1.3[a,c,d]	7.5 ± 0.7[b,c,d]	5.4 ± 1.2[b,c,d]	4.4 ± 0.4[a,c,d]
	DHA-EE	1.9 ± 0.3[a,e]	1.5 ± 0.7[a,e]	1.6 ± 0.7[a,e]	1.2 ± 0.1[a]
22:5n-3	Palm	1.0 ± 0.2[a]	1.1 ± 0.1[a]	1.2 ± 0.2[a]	1.2 ± 0.1[a]
	EPA-EE	1.8 ± 0.2[a,d]	2.8 ± 0.1[b,c,d]	2.8 ± 0.7[b,c,d]	3.0 ± 0.6[b,c,d]
	DHA-EE	1.4 ± 0.3[a]	1.4 ± 0.1[a]	1.2 ± 0.1[a]	1.3 ± 0.1[a]
22:6n-3	Palm	7.8 ± 0.6[a]	7.2 ± 0.8[a]	7.9 ± 1.1[a]	7.0 ± 1.4[a]
	EPA-EE	7.2 ± 0.5[a,c]	7.2 ± 0.5[a,c]	6.9 ± 0.8[a,c]	7.0 ± 0.5[a,c]
	DHA-EE	9.7 ± 1.1[a,e]	9.7 ± 1.5[a,e]	8.8 ± 0.6[a]	10.1 ± 0.3[a,e]
Σ monoenes	Palm	13.5 ± 1.9[a]	14.7 ± 1.8[a]	12.4 ± 1.5[a]	15.6 ± 5.2[a]
	EPA-EE	12.5 ± 0.8[a]	11.3 ± 0.5[a,c,d]	11.4 ± 1.2[a]	12.0 ± 1.0[a]
	DHA-EE	12.9 ± 0.8[a]	14.8 ± 1.4[a]	13.6 ± 1.3[a]	11.9 ± 0.6[a]
Σ polyenes	Palm	44.4 ± 3.2[a]	41.6 ± 1.8[a]	45.7 ± 0.8[a]	41.8 ± 4.4[a]
	EPA-EE	45.5 ± 2.5[a]	49.1 ± 1.2[a,c,d]	48.8 ± 1.7[a,c,d]	44.3 ± 0.8[a]
	DHA-EE	46.0 ± 1.6[a]	43.8 ± 1.4[a]	42.6 ± 2.4[a]	47.7 ± 2.5[a]
n-3/n-6	Palm	0.30 ± 0.03[a]	0.30 ± 0.03[a]	0.29 ± 0.05[a]	0.30 ± 0.03[a]
	EPA-EE	0.42 ± 0.03[a,c,d]	0.58 ± 0.07[b,c,d]	0.47 ± 0.08[a-d]	0.40 ± 0.07[a]
	DHA-EE	0.41 ± 0.05[a,e]	0.42 ± 0.08[a,e]	0.38 ± 0.04[a]	0.37 ± 0.04[a]

[a,b]Values in a row with different superscripts are significantly different ($P < 0.05$). Abbreviations as in Table 2.
[c]$P < 0.05$ for difference between EPA-EE and DHA-EE treated rats.
[d]$P < 0.05$ for difference between EPA-EE and controls.
[e]$P < 0.05$ for difference between DHA-EE and controls.

The results from this study demonstrated that both EPA-EE and DHA-EE administration resulted in the induction of fatty acyl-CoA oxidase activity (Table 2). As the fatty acyl-CoA oxidase mRNA levels were increased (Vaagenes, H., Asiedu, D., Demoz, A., and Berge, R.K., unpublished results), the induction of fatty acyl-CoA oxidase by these peroxisome proliferators is partly, if not entirely, due to an increase in the transcription rate of the gene. EPA-EE lowered plasma triglycerides, but DHA-EE supplementation had no hypotriglyceridemic effect. Thus, increased triglyceride clearance by fish oil is not due to enhanced activity of peroxisomal β-oxidation, which confirms and extends previous reports by hypolipidemic peroxisome proliferating fatty acid analogues (71).

To our knowledge, the results from this study demonstrate for the first time that EPA-EE (but not DHA-EE) treatment results in the induction of rat liver 2,4-dienoyl-CoA reductase gene expression, an enzyme which is necessary for oxidation

TABLE 5
HMG–CoA Reductase Activity at Different Times After Acute EPA-EE and DHA-EE Administration in the Rat[a]

	Hours	EPA-EE	*P*	Control	*P*	DHA-EE
HMG–CoA	2	184 ± 98[a]	n.s.	91 ± 16[a]	<0.05	240 ± 22[a]
reductase activity	4	241 ± 34[a]	n.s.	150 ± 47[a]	n.s.	228 ± 22[a]
(pmol/min/mg protein)	8	152 ± 54[a]	<0.05	237 ± 40[b]	<0.05	335 ± 17[b]
	12	186 ± 129[a]	n.s.	269 ± 79[b]	n.s.	266 ± 79[a,b]
	24	344 ± 59[a]	n.s.	233 ± 97[a,b]	n.s.	270 ± 22[a]

[a]The 3-hydroxy-3-methylglutaryl (HMG)–CoA reductase activity was measured in the isolated microsomal fraction. Statistical comparison between EPA-EE, DHA-EE, and controls is shown by the *P* values.
[a,b]Correspond to the statistical comparison between the groups at each of the time points studied (hours), where the same letter within one column means that there is no statistical differences between the corresponding groups. Abbreviations as in Table 2.

of unsaturated fatty acids. Furthermore, the induced activity of 2,4-dienoyl-CoA reductase on the transcriptional induction of the enzyme gene expression by EPA-EE supports previous reports that modification of the mitochondrial fatty acid oxidation system is related to the mitochondrial induction produced by non-β-oxidizable fatty acid analogues, i.e., 3-thia fatty acids (71) and by fish oil (72). Increased fatty acid oxidation of EPA, but not DHA (free forms of the n-3 fatty acids), seen in cultured rat hepatocytes (Fig. 1) is in keeping with the animal data. Thus, the triglyceride-lowering effect of EPA may be due to the increased mitochondrial fatty acid oxidation.

McGarry and Foster (73) have shown that fatty acid oxidation is inhibited by malonyl-CoA, *via* inhibition of carnitine palmitoyltransferase I. Depression of the hepatic malonyl-CoA content (Fig. 4C) therefore appears to be the mechanism by which EPA stimulates mitochondrial fatty acid oxidation. It is tempting to suggest that EPA, by decreasing malonyl-CoA, may promote acyl carnitine formation and, consequently, acylcarnitine transport across the inner mitochondrial membrane followed by β-oxidation of long-chain acyl-groups to acetyl-CoA.

A number of animal studies have demonstrated increased hepatic β-oxidation capacity and ketone body production after fish oil feeding (17,19,25,74,75). Surette *et al.* (20) observed an increased activity of hepatic carnitine palmitoyl transferase I in fish oil-fed Syrian hamsters, which was highly correlated with the diet-induced change in serum triacylglycerol concentrations. These findings are in agreement with the results in this study and previous findings (21,22), and one reason for the different results with EPA and DHA might be that the bioavailability of EPA and DHA for the mitochondrial and peroxisomal β-oxidation is different. Very long chain fatty acids (VLCFA) (>C_{20}) are oxidized predominantly, if not exclusively, by peroxisomes (76,77) and very long chain fatty acyl-CoA synthetase (necessary for the activation of the fatty acids to their CoA derivatives, a prerequisite for β-oxidation and esterification) is present in endoplasmic reticulum and peroxisomes, but not in mitochondria (77,78). Apparently, the site of activation determines the subsequent fate of VLCFA. VLCFA are not oxidized by isolated mitochondria, indicating that the absence of very long chain acyl-CoA synthetase from mitochondria is the reason why these organelles do not oxidize VLCFA (79). Because DHA is a good substrate for peroxisomes and increases peroxisomal β-oxidation, but not the mitochondrial β-oxidation (Table 3 and Fig. 4), we suggest that DHA is mainly metabolized (chain-shortened/retroconverted) by peroxisomes. This is in agreement with studies in isolated liver cells (80). In contrast, EPA and other long-chain fatty acids (LCFA; C_{16}–C_{20}) are mainly directed to mitochondrial β-oxidation, as long-chain acyl-CoA synthetase (C_{16}–C_{20}) is located in mitochondria and they have a lower apparent K_m for LCFA (81,82). The different results of EPA and DHA on triglyceride-lowering effect and hepatic fatty acid oxidation may partly reflect the diversion of EPA (as a LCFA) to mitochondrial β-oxidation and ketogenesis, whereas DHA is diverted to the peroxisomes for further metabolism.

Circulating FFA and glycerol are mainly taken up by the liver where they are partially converted into triglycerides for later export to the circulation in the form of VLDL-triglycerides. Several studies have reported a marked reduction in FFA concentration after fish oil supplementation, indicating that decreased lipolysis from adipose tissue could be the reason for decreased VLDL synthesis and/or secretion (13,75, 83). However, the activities of lipoprotein lipase and hepatic lipase have uniformly shown no change after fish oil feeding both in humans (84,85) and in animals (86,87). Otto *et al.* (88) observed a dose-dependent reduction in plasma FFA by n-3 fatty acids in rats fed *ad libitum*, but not in rats fasted overnight (88). In the present study, administration of EPA-EE reduced the plasma FFA at eight hours, whereas plasma FFA in DHA-EE treated animals showed a marked decrease in plasma FFA after only two hours. At that time the plasma FFA in EPA-EE treated rats were significantly higher than in DHA-EE treated rats and controls (Fig. 3B). A possible explanation for this might be the recently published observations by Raclot and Groscolas (89) that individual fish oil n-3 polyunsaturated fatty acids are selectively mobilized from and stored in adipose tissue. The preferential mobilization of EPA could contribute to its maintenance in the circulation at four hours compared to DHA, and that this effect was overcome at later time points.

In general, hepatic fatty acid oxidation is proportional to plasma FFA concentration (90) but, at equal FFA concentrations, perfused livers from fish oil-adapted rats have shown

significantly higher rates of ketogenesis than livers from animals fed chow or safflower oil (19). This is in agreement with our results where all three fatty acid supplements resulted in decreased concentrations of plasma FFA at eight hours, but at the same time point mitochondrial β-oxidation was significantly increased in the EPA-EE treated rats (Fig. 4). This supports our data that decreased plasma triglycerides following EPA-EE supplementation is due to increased mitochondrial β-oxidation, resulting in decreased amount of substrate for triglyceride synthesis, and is not due to increased lipolytic activity.

There is no consistent change in plasma cholesterol after fish oil feeding in humans, whereas the cholesterol-lowering effect of fish oil in rats is well documented (29,31). One explanation might be that rats are deficient in lipid transfer protein (91) and in this species fish oils lower plasma cholesterol concentrations, probably as a consequence of this lack. In humans and marmosets who have lipid transfer protein, there may be even a small increase in plasma cholesterol after ingestion of fish oil and EPA (as ethyl ester), respectively (92,93). Human studies also have demonstrated that fish oil reduces the expected increase in plasma cholesterol levels with an atherogenic diet (94). As with the hypotriglyceridemic effect, it remains unknown whether EPA or DHA or both are responsible for the cholesterol-lowering effect in rats. In this and a previous study, we have observed a hypocholesterolemic effect of EPA-EE, and not DHA-EE, associated with a significant inhibition of HMG–CoA reductase activity (Table 5) (22). This suggests that EPA-EE, in contrast to DHA-EE treatment, might decrease cholesterol biosynthesis, thereby contributing to the decrease in VLDL secretion.

Another important observation made in this study was that EPA-EE, but not DHA-EE, given acutely affected the cellular fatty acid composition by decreasing the amounts of monoenes (16:1n-7, 18:1n-7, and 18:1n-9) and increasing the amount of polyenes (20:5n-3 and 22:6n-3). Replacement of the ordinary fatty acids, i.e., the monoenes with EPA and some conversion to DPA concomitant with increased fatty acid oxidation, is probably the mechanism leading to changed fatty acid composition. The paradox that administration of pure EPA-EE did not increase DHA concentration in liver (Table 4) may be explained by reduced Δ^6 desaturase activity as 22:5n-3 is metabolized to 22:6n-3 through three steps: namely elongation to 24:5n-3, Δ^6-desaturation to 24:6n-3, and finally β-oxidation to DHA (95). Indeed, dietary supplementation with n-3 fatty acids depresses rat liver Δ^6-desaturase activity (96), which is necessary for the second step of the conversion of 22:5n-3 to 22:6n-3.

The results from this study argue against a direct conversion of EPA to DHA, but rather in favor of retroconversion of EPA to DHA (Table 4). Retroconversion of DHA to EPA also has been reported by others (23,27). This implies that it will be difficult to study the unique effects of DHA as some of it will always be converted to EPA. Whether the effects observed in the DHA-EE treated group were due to the EPA generated from DHA, and not from DHA itself, must then be considered.

In conclusion, we have shown that EPA is the hypotriglyceridemic fatty acid in fish oils, and increased mitochondrial fatty acid oxidation may be responsible. DHA has little impact on serum lipid levels, but can be retroconverted to EPA. In summary, the present observations provide evidence for the following events in the liver following exposure to EPA: (i) inhibition of malonyl-CoA formation possibly due to increased long-chain acyl-CoA levels (Fig. 4) (most plausible attributed to EPA-CoA); (ii) increased mitochondrial fatty acid oxidation; (iii) incorporation into triglycerides and phospholipids and displacement of easily oxidizable fatty acids into β-oxidation in mitochondria; and (iv) decreased production of VLDL-triglycerides.

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Δ8 Desaturation *in vivo* of Deuterated Eicosatrienoic Acid by Mouse Liver

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ABSTRACT: *In vitro* evidence has been reported for an alternate pathway that involves Δ8 desaturation of n-6 and n-3 polyunsaturated fatty acids (PUFA). The present study was designed to allow detection of Δ8 desaturation *in vivo* and to provide an estimation of the relative contribution of Δ8 desaturation to the *in vivo* synthesis of n-3 fatty acids. Male adult ICR mice were fed a semisynthetic fat-free diet for eight days, and then the diets were supplemented for three days with deuterated 11,14,17-eicosatrienoic acid (20:3-d_8) labeled at the 3,3,4,4,8,8,9,9 carbon positions. Analysis of liver total lipid by gas chromatography/mass spectroscopy indicates that the total deuterated fatty acids contained 22.3% 20:3n-3-d_8 and 28.9% of metabolites formed by elongation and Δ5 desaturation of 20:3n-3-d_8. Deuterated metabolites resulting from retroconversion to 18:3-d_6 and subsequent metabolism by classical pathways represented 35.3% of the total deuterated fatty acids. The retroconversion product (18:4n-3-d6) of 20:4n-3-d_6 and/or -d_8 was 9.0% of the total. A minor percentage (4.4%) of the products identified (20:4n-3-d_6, 20:5n-3-d_6, 22:5n-3-d_6, 22:6n-3-d_5 and 24:6n-3-d_5) were formed by Δ8 desaturation. This study provides the first clear evidence of Δ8 desaturation *in vivo* in the mouse liver. Whether Δ8 desaturation would have a greater importance *in vivo* when the Δ6 desaturase pathway is disrupted remains to be determined.

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All mammals require the presence of both n-6 and n-3 polyunsaturated fatty acids (PUFA) in the diet. The essential fatty acids, linoleic (18:2n-6) and linolenic (18:3n-3), are desaturated and elongated by a series of reactions, forming longer chain PUFA (1). The major end-products of linoleic acid metabolism are primarily arachidonic acid (20:4n-6) and docosapentaenoic acid (22:5n-6), and the major metabolites derived from linolenic acid are eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3).

The classical pathway of PUFA formation from linolenic acid involves a series of desaturation and 2-carbon chain elongation steps. Starting with a Δ6 desaturation, the first product is 18:4n-3. A chain elongation then occurs producing 20:4n-3, followed by a Δ5 desaturation producing 20:5n-3. Another chain elongation takes place to form 22:5n-3 and is followed by a Δ4 desaturation step to produce 22:6n-3. This same series of reactions is responsible for the formation of 22:5n-6 from linoleic acid (2,3). An alternate pathway for the formation of 22:6n-3 and 22:5n-6 has been described (4). In this pathway, 22:5n-3 or 22:4n-6 is elongated to 24:5n-3 or 24:4n-6, and then Δ6 desaturated to produce 24:6n-3 or 24:5n-6, respectively. A 2-carbon chain shortening then occurs, forming 22:6n-3 or 22:5n-6.

Although both enzymatic studies and results from rats fed and injected with carbon-14 labeled n-6 and n-3 PUFA have led to the conclusion that 8-desaturation does not occur in rat liver (5,6), a number of other studies have reported both direct and indirect evidence that 8-desaturation is an alternate pathway for formation of n-6 and n-3 PUFA from linolenic and linoleic acid. Rat testes injected with [1-^{14}C]eicosa-11,14-dienoic acid resulted in the appearance of radiolabeled eicosa-8-11-14-trienoic acid, providing initial evidence for the existence of Δ8 desaturation (7). This study was later substantiated by a similar study with human testicular tissue (8). Cultured rat C_6 glioma cells have been shown to produce eicosapentaenoic acid (20:5n-3) when 11,14,17-20:3-8,8,9,9-d_4 was added to the medium (9), and rat C_6 glioma, mouse neuroblastoma (N1E-115), human neuroblastoma (HTB-10, HTB-11), and human infant male foreskin fibroblast (F8) cells have shown Δ8 desaturase activity *in vitro* when 11,14,17-20:3-3,3,4,4,8,8,9,9-d_8 was added to media (10). Studies with human cancer and noncancerous cells indicated that substantial conversion of 11,14-20:2 to 20:4n-6 occurred *in vitro* (11). The fatty acid composition of liver lipids from rats fed 5,11,14,17-20:3 (12) and of human leukemia cells that lack Δ6 desaturase were also consistent with the possibility of Δ8 desaturation (13).

The present study was designed to investigate whether Δ8 desaturation of n-3 fatty acids occurs *in vivo* and to determine if Δ8 desaturation contributes to the *in vivo* synthesis of n-3 fatty acids. The specific labeling pattern for the 20:3n-3-d_8 substrate used was selected to allow differentiation between the deuterated n-3 fatty acids formed by retroconversion of 20:3n-3-d_8 and subsequent conversion to n-3 fatty acids by the classical pathway and deuterated n-3 fatty acids formed by an 8-desaturation pathway. The presence of any d_6 n-3 fatty acid products would be evidence of 8-desaturation since 4 or 6 deuterium labels would be lost by any other pathways.

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Abbreviations: EFA, essential fatty acid; FF, fat-free; FFS, fat-free supplemented; GC, gas chromatography; MS, mass spectroscopy; PUFA, polyunsaturated fatty acids.

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MATERIALS AND METHODS

Animals and diets. Male adult (approximately 7 mon old) ICR mice (Sprague-Dawley, Indianapolis, IN) of similar body weight (approximately 45 g) were used in this study. Mice were housed individually, with a 12 h light/12 h dark cycle, an ambient temperature of 22°C, and controlled humidity. Purina Laboratory Rodent Chow 5001 (Purina Mills, Richmond, IN) and water was available *ad libitum* prior to the feeding of the experimental diet. Food consumption was monitored, and body weight recorded weekly.

For the experiment, seven mice were fed a semi-synthetic fat-free (FF) diet (7 g/d) containing 65% sucrose, 20% vitamin-free casein, 3.5% salt mixture (AIN Mineral Mixture 76), 2.2% vitamin mixture, 0.3% methionine, and 9.0% cellulose for 8 d. Dietary ingredients were purchased from Sigma Chemical Co. (St. Louis, MO) and ICN Nutritional Biochemicals (Cleveland, OH). Three mice were maintained on this FF diet for an additional three days as a control group—FF and the diets of four mice were supplemented with 30 mg/d deuterated 11,14,17-eicosatrienoic acid (20:3n-3-d_8) labeled at the 3,3,4,4,8,8,9,9 carbon positions [fat-free supplemented (FFS)], for three days. Methyl esters of 11,14,17-20:3-d_8 were prepared by previously described synthetic methods (14). Chemical purity was 99.8%, and isotopic purity was 7.9 deuterium atoms per molecule (4.0% d_7, 93.5% d_8, 2.4% d_9). Analysis of samples extracted from unused test diet two hours after exposure to air at room temperature indicated that oxidation was not a problem and that the saponification and esterification reactions did not cause loss or scatter of deuterium or the formation of fatty acid isomers. The free fatty acid of 11,14,17-20:3-d_8 was prepared for feeding by saponification (15), and stored at −20°C under nitrogen until feeding; 30 mg of 11,14,17-20:3-d_8 free fatty acid were diluted with 1 mL of anhydrous ether (J.T. Baker Inc., Phillipsburg, NJ), and added to the individually weighed portion of diet daily. The ether was removed by evaporation, and the diet immediately fed. Mice were fed late in the afternoon shortly before the start of the dark cycle. All diet was normally consumed within two hours of feeding.

Sample collection. At the end of the feeding period, mice were euthanized by halothane (Halocarbon Laboratories, Inc., North Augusta, SC) overdose, and livers were excised, rinsed with cold 0.9% saline, blotted dry, placed in tared vials, and weighed. Livers were stored in chloroform/methanol (2:1, vol/vol) under nitrogen at −20°C until extracted and processed for fatty acid analysis.

Extraction. Liver samples (approximately one gram) were homogenized in 40 mL chloroform/methanol (2:1, vol/vol) for approximately 30 s (Tissumizer, Model #SDT1810; Telmar, Cincinnati, OH) and filtered. Liver lipids were extracted according to previously described procedures (16). Heptadecanoic acid (17:0; Nu-Chek-Prep, Elysian, MN) was added to lipid extracts as an internal standard. Lipid extracts were esterified with 4% H_2SO_4/methanol according to standard procedures (15).

Analysis of fatty acids. The fatty acid composition of liver tissue was determined using a Hewlett-Packard 5890 Series II GC (Hewlett-Packard, Avondale, PA) equipped with a flame-ionization detector and a 0.25 mm × 30 m fused silica SP2330 capillary column (0.2 μm film thickness; Supelco, Inc., Bellefonte, PA). The initial temperature of the column was 165°C for an initial time of 19 min; the temperature was then increased to 210°C at the rate of 1°C/min. Helium was used as the carrier gas (linear flow rate = 19.2 cm/s), and the injector and detector temperatures were 245 and 250°C, respectively. Fatty acids were identified by comparison of retention times to those of authentic standards (Nu-Chek 68A Standard to which known weights of 17:0, 20:3n-9, 20:5n-3, 22:3n-6, 22:4n-6, and 22:5n-6 had been added; Nu-Chek-Prep). Peak areas were measured using an HP 3396A Integrator (Hewlett-Packard), and compared to the internal 17:0 standard peak area.

Liver methyl esters were also analyzed by gas chromatography/mass spectrometry (GC/MS) using a modification of a previously published method (17). The GC/MS system used was a Hewlett-Packard 5980 Series II GC/5988A Research Grade MS/Pascal 59970C Chem Station with a Supelcowax-10 (30 m × 0.25 mm, 0.25 μm film thickness) column (Supelco, Inc.). The GC conditions were: initial column temper ature 70°C; initial hold 1 min; program rate 1, 30°C/min to 190°C; program rate 2, 4°C/min to 232°C; program rate 3, 3.5/min to a final temperature of 260°C; injection temperature 265°C. The flow rate of the helium carrier gas was 0.91 mL/min. Splitless injection was used. The mass spectrometer conditions were: filament voltage 70 ev; filament emission current 230 ma; multiplier setting 1800 to 2400 volts; source temperature 200°C; isobutane pressure 1.0 torr. Selected-ion monitoring of ions with even masses was used for quantitative analysis except when qualitative analysis had identified the presence of fatty esters with an odd-number of deuterium labels.

The Nu-Chek 68A standard mixture containing added fatty esters (described earlier) was used to calculate GC/MS response factors. The response factors were updated before and after the analysis of each series of four samples. Methyl esters from *Ephedra campylopoda* seed oil were used to determine retention times and response factors for 5*c*,11*c*,14*c*-20:3 and 5*c*,11*c*,14*c*,17*c*-20:4 (5*c*20:4n-3) (18). The response factor for 4,7,10,13,16,19-22:6 was used as the response factor for 9,12,15,18,21-24:5 and 6,9,12,15,18,21-24:6.

Statistical analysis of data. The Student's *t*-test was used to determine significant differences in body weight, tissue weight, and liver fatty acid content. Statistical procedures were performed using NWA Statpak, version 4.1 (Northwest Analytical, Inc., Portland, OR) on an IBM compatible computer.

RESULTS

Animal data. There was no significant difference in the amount of diet consumed between the FF diet-fed mice and

the 20:3n-3-d_8 FFS mice. Also, there were no significant differences in body weights (45.7 ± 3.6 g) or total liver weights (2.5 ± 0.56 g) at the end of the feeding period between the mice fed 20:3n-3-d_8. Weights for the total liver lipid extracts (29.5 ± 3.55 mg/g) were also similar for both groups of mice. Percentages of liver lipids in FF mice were in agreement with previously published values (19).

Liver fatty acid profile. The fatty acid profile of liver lipids for both FF mice and FFS mice is shown in Table 1. In both FF and FFS mice, saturated and monoenoic fatty acids accounted for greater than 80% of the total liver fatty acids (81 and 83%, respectively). Liver lipids from FFS mice contained 4.4 and 4.9% 18:2n-6, respectively. Predominant PUFA present in both FF and FFS mouse livers were 20:4n-6 (4.5 and 5.7%, respectively) and 22:6n-3 (5.0 and 5.1%, respectively). The 1.1 to 1.2% of 20:3n-9 in the livers from both groups of mice indicate that the short-term lack of dietary essential fatty acids (EFA) had not caused a large increase in this marker for EFA deficiency. There was no significant difference in the presence of any of the n-6 series of PUFA in FF or FFS mouse livers. However, 20:3n-3-d_8 supplementation resulted in significantly higher concentrations of 18:4n-3, 20:3n-3, 5*c*20:4n-3, and 7*c*,13*c*,16*c*,19*c*-22:4n-3 (7*c*22:4n-3) in liver, primarily due to the presence of deuterated forms of these fatty acids.

TABLE 1
Liver Fatty Acid Profile of Fat-Free Fed (FF) and Fat-Free Fed, 20:3n-3-d_8 Supplemented (FFS) Mice

Fatty acyl chain	FF[a]	FFS[b]
14:0	104.9 ± 15.1	119.5 ± 39.4
14:1	8.4 ± 3.5	6.6 ± 2.9
16:0	4970.5 ± 859.6	5793.0 ± 1139.0
16:1	939.2 ± 336.7	851.0 ± 267.8
17:1	29.5 ± 6.5	37.2 ± 11.4
18:0	1945.6 ± 548.2	1763.0 ± 372.0
18:1n-7 and n-9	5301.1 ± 1674.3	5720.9 ± 1854.3
18:2n-6	718.6 ± 182.3	885.1 ± 247.4
18:3n-3	6.7 ± 2.2	21.0 ± 7.6
18:3n-6	6.0 ± 1.9	8.6 ± 1.3
18:4n-3	0.2 ± 0.2[c]	10.2 ± 3.6[c]
20:0	34.7 ± 17.8	27.1 ± 9.75
20:1n-9	129.7 ± 39.2	98.1 ± 36.3
20:2n-6	22.2 ± 11.2	14.2 ± 3.8
20:3n-3	10.8 ± 3.6[d]	46.1 ± 8.8[d]
20:3n-6	164.1 ± 56.2	161.8 ± 45.8
20:3n-9	191.1 ± 87.6	208.0 ± 87.3
5*c*20:4n-3	9.3 ± 5.2[c]	41.6 ± 9.4[c]
20:4n-3	8.5 ± 2.6	8.6 ± 1.4
20:4n-6	739.0 ± 271.6	1026.9 ± 177.4
20:5n-3	44.7 ± 19.4	52.2 ± 22.6
22:0	20.6 ± 6.9	14.4 ± 4.8
22:1n-9	13.7 ± 7.4	6.8 ± 2.0
22:2	1.2 ± 0.6	1.0 ± 0.4
22:3n-3	0.2 ± 0.2	1.4 ± 0.5
7*c*22:4n-3	0.6 ± 0.4[c]	13.1 ± 5.4[c]
22:4n-6	18.7 ± 9.9	23.0 ± 3.5
22:5n-3	16.5 ± 6.3	21.8 ± 4.3
22:6n-3	809.0 ± 432.4	921.1 ± 193.8
14:0	15.1 ± 4.7	5.6 ± 1.2
24:1	17.4 ± 7.2	10.1 ± 1.3
24:6n-3	ND[e]	0.2 ± 0.1
Total	16317.2 ± 3689.4	17937.2 ± 4428.5

[a]Mean μg/g ± SEM, $n = 3$.
[b]Mean μg/g ± SEM, $n = 4$.
[c]$P < 0.05$.
[d]$P < 0.01$.
[e]ND = None detected.

Metabolism of deuterated 20:3n-3-d_8. Supplementation of an FF diet with 11,14,17-20:3-d_8 for three days resulted in the incorporation into mouse liver of a number of metabolites formed from 20:3n-3-d_8. Portions of some of the selected ion tracings obtained by GC/MS are shown in Figures 1 and 2 in order to qualitatively illustrate the separations and the signal-to-noise ratios. The combination of both GC retention times and molecular ion data greatly reduces the possibility of misidentification of the d_2, d_4, d_5, d_6, and d_8 products from 20:3n-3-d_8. For example, 5*c*20:4n-3-d_6 with a mass of 325 was easily distinguished from 5*c*20:4n-3-d_8 with a mass of 327, and the small amount of 20:4n-3-d_4 present with a mass of 323 could be detected separately from 20:4n-3 with a mass of 319 (Fig. 1A). Using this method, 22:6n-3-d_5 with a mass of 348 could be identified separately from 22:6n-3-d_4 with a mass of 347 (Fig. 1B). In Figure 2, the *m/z* 325 and *m/z* 323 ion scans have been expanded to show GC/MS data for the small amounts of 20:4n-3-d_6 and 20:5n-3-d_6 product obtained by Δ8 desaturation. The slightly earlier elution of the deuterated fatty acids relative to the undeuterated fatty acids was also used to confirm identifications. No peaks were found in samples of liver fatty methyl esters from the mice fed the nonsupplemented FF control diet that would coelute with any of the identified d_4, d_5, d_6, and d_8 PUFA peaks.

Supplementation of an FF diet with 20:3n-3-d_8 resulted in the production of a number of deuterated PUFA. The quantitative data for the 20:3n-3-d_8 substrate that was fed and the deuterated products identified in the liver lipids are listed in Table 2. Deuterated fatty acids accounted for 0.6% of the total liver fatty acids. The fatty acid fed, 20:3n-3-d_8, was present in the highest quantity in the liver (22.3% of total deuterated fatty acids). Major deuterated metabolites of 20:3n-3-d_8 were 5*c*20:4n-3-d_8 (20.6% of total deuterated fatty acids), 18:4n-3-d_6 (9.0%), 18:4n-3-d_4 (8.6%), 7*c*22:4n-3-d_8 (7.6%), and 18:3n-3-d_6 (5.4%). The deuterated metabolites formed from Δ8 desaturation of 20:3n-3-d_8 were 20:5n-3-d_6, 22:5n-3-d_6, 22:6n-3-d_5, and 24:6n-3-d_5. The sum for these deuterated fatty acids was 4.4% of the total deuterated fatty acids.

DISCUSSION

The present evaluation of the metabolism of 20:3n-3 has been facilitated by the availability of this fatty acid labeled with deuterium atoms at specific locations in the fatty acyl chain. Selective mass detection allowed a variety of metabolites derived from 20:3n-3-d_8 to be identified and quantified (17).

The classical pathway for synthesis of 20-carbon PUFA from 18:2n-6 and 18:3n-3 occurs by an alternating sequence of desaturation and elongation reactions has long been established (5,20). The feeding of 20:3n-3-d_8 resulted in the incorporation of 23.6 μg/g of 20:3n-3-d_8 per gram of liver, repre-

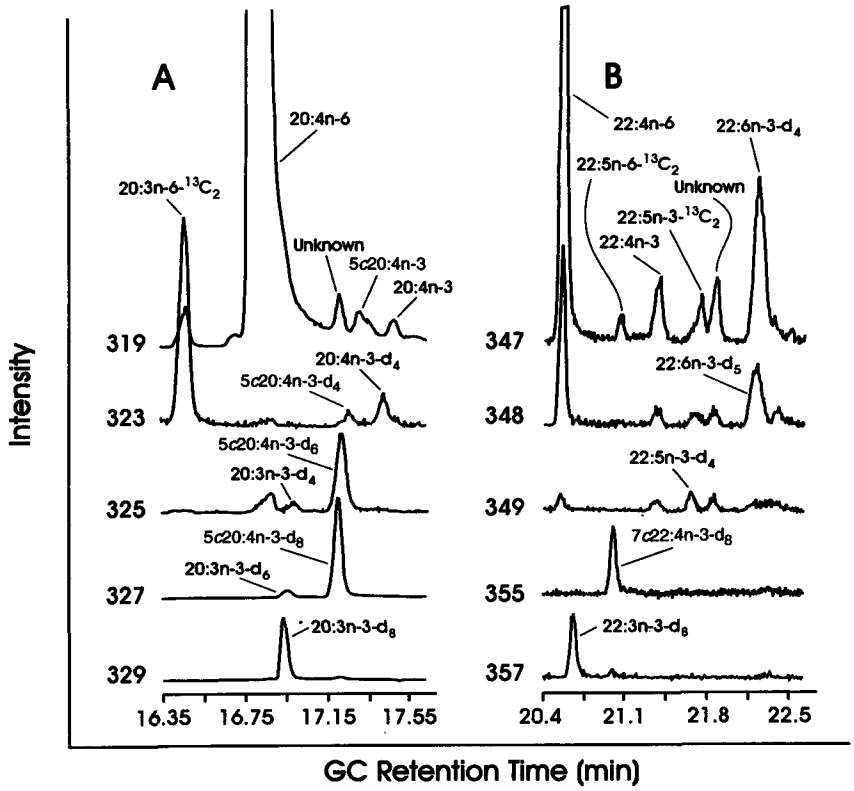


FIG. 1. Selected gas chromatography (GC)/mass spectrometry ion scans for methyl esters of liver total lipids from mouse fed 20:3n-3-d_8: A: 20-carbon fatty esters, B: 22-carbon fatty esters. The selected ion scans have been manually scaled to minimize overlap between tracings and should be used only for qualitative purposes.

senting 22.3% of total deuterated fatty acids. Classical metabolites of 20:3n-3-d_8 were present, and accounted for 28.9% of the total deuterated fatty acids detected (Scheme 1). These metabolites included 22:3n-3-d_8, 5*c*20:4n-3-d_8, and 7*c*22:4n-3-d_8. The metabolite 22:3n-3-d_8 is formed *via* an initial 2-carbon chain elongation of 20:3n-3-d_8. The metabolite 5*c*20:4n-3-d_8 is formed after a Δ5 desaturation of 20:3n-3-d_8, resulting in no loss of deuterium atoms; 7*c*22:4n-3-d_8 is produced after the 2-carbon chain elongation of 5*c*20:4n-3-d_8.

A number of deuterated metabolites also resulted from apparent retroconversion and subsequent classical pathway metabolism of 20:3n-3-d_8 (Scheme 2). These metabolites accounted for 35.3% of the total deuterated fatty acids observed. *Via* retroconversion, 18:3n-3-d_6 is produced by the loss of 2-carbons and the 2 deuterium atoms at the 3 position. Once 18:3n-3-d_6 is formed, either a 2-carbon chain elongation can occur producing 20:3n-3-d_6, or Δ6 desaturation can result in the formation of 18:4n-3-d_4 since two deuterium atoms are lost in this desaturation. The 20:3n-3-d_6 can undergo the classical Δ5 desaturation, producing 5*c*20:4n-3-d_6, and then a subsequent 2-carbon chain elongation, forming 7*c*22:4n-3-d_6. The 18:4n-3-d_4 produced *via* Δ6 desaturation can undergo a sequence of 2-carbon chain elongations and desaturations (Δ5, Δ4) producing 20:4n-3-d_4, 20:5n-3-d_4, 22:5n-3-d_4, 22:6n-3-d_4, and 24:6n-3-d_4. Alternately, 22:5n-3-d_4 may be elongated to 24:5n-3-d_4 and then undergo Δ6 desaturation and retroconversion to produce 22:6n-3-d_4. No deuterium atoms are lost in this sequence of elongations and desaturations.

Retroconversion of 11,14,17-20:3-3,3,4,4,8,8,9,9-d_8 also resulted in a small quantity of 18:3n-3-6,6,7,7-d_4 formation *via* the loss of four deuterium atoms (Scheme 3). One possible reason for why some deuterium-label at the C4 position is lost is because the intermediate formed during β-oxidation has an enol structure that allows hydrogen–deuterium exchange to occur at the 4 position. Another possibility is that a second β-oxidation cycle produced 7,10,13,16-16:3n-3-d_4 which was then reelongated to 18:3n-3-d_4. This second explanation is consistent with the deuterium distribution pattern for 18:3n-3. Mostly d_4 and d_6 18:3n-3 were found, and there was very little of the 18:3n-3-d_5 product that would be formed by an exchange mechanism. The 16:3n-3-d_4 was detected which would be consistent with the second possibility. The 18:4n-3-d_2, 20:3n-3-d_4, and 20:4n-3-d_4 products can be formed from 18:3n-3-d_4 by the previously described pathways.

Several deuterated metabolites were identified which could not be synthesized by the classical fatty acid metabolism or retroconversion pathways. A possible explanation for the presence of these metabolites involves a Δ8 desaturation in the metabolic pathway of 20:3n-3-d_8 (Scheme 4). The feed-

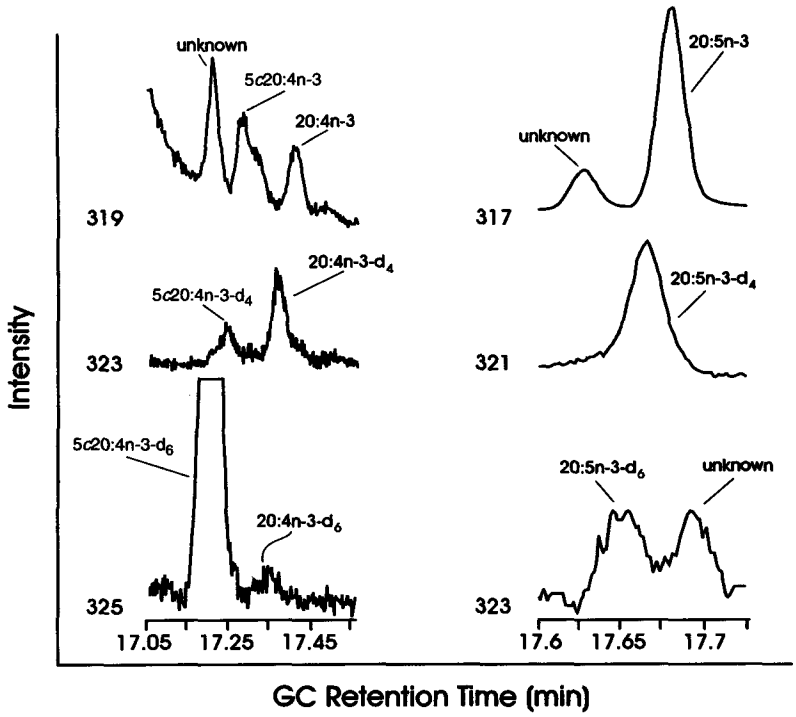


FIG. 2. Selected gas chromatography/mass spectrometry ion scans for the 20:4n-3-d_6 and 20:5n-3-d_6 fatty esters from total liver lipids of a mouse fed 20:3n-3-d_8. The selected ion scans have been manually scaled to minimize overlap between tracings and should be used only for qualitative purposes. See Figure 1 for abbreviation.

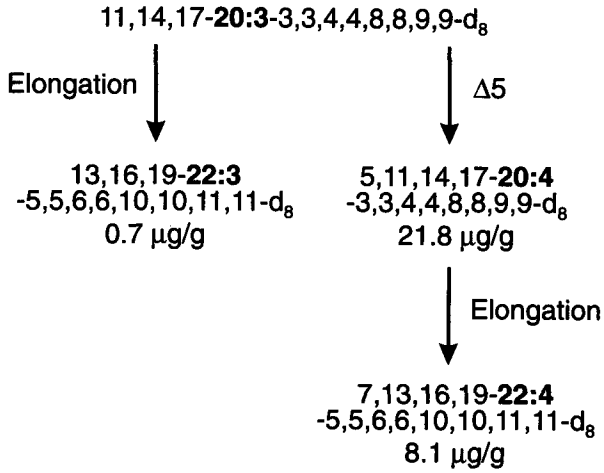
TABLE 2
Deuterated Metabolites in the Livers of Mice Fed a Fat-Free Diet Supplemented with 20:3n-3-d_8

Fatty acyl chain	μg/g	%Total deuterium
18:3n-3-d_4	3.8 ± 1.9[a]	3.6 ± 0.8[b]
18:3n-3-d_6	5.7 ± 3.2	5.4 ± 1.5
18:4n-3-d_2	0.4 ± 0.2	0.4 ± 0.2
18:4n-3-d_4	9.1 ± 3.2	8.6 ± 3.5
18:4n-3-d_6	9.5 ± 2.9	9.0 ± 3.1
20:3n-3-d_4	1.7 ± 0.2	1.6 ± 0.3
20:3n-3-d_6	2.2 ± 0.7	2.1 ± 0.1
20:3n-3-d_8	23.6 ± 9.4	22.3 ± 2.9
5*c*20:4n-3-d_4	0.8 ± 0.1	0.8 ± 0.2
5*c*20:4n-3-d_6	1.8 ± 0.6	1.7 ± 0.2
5*c*20:4n-3-d_8	21.8 ± 7.9	20.6 ± 2.9
20:4n-3-d_4	0.8 ± 0.2	0.8 ± 0.2
20:4n-3-d_6	0.05 ± 0.01	0.05 ± 0.01
20:5n-3-d_4	3.9 ± 0.9	3.7 ± 1.1
20:5n-3-d_6	1.0 ± 0.4	0.9 ± 0.5
22:3n-3-d_8	0.7 ± 0.5	0.7 ± 0.2
7*c*22:4n-3-d_4	1.5 ± 0.5	1.4 ± 0.9
7*c*22:4n-3-d_6	3.5 ± 1.5	3.3 ± 2.6
7*c*22:4n-3-d_8	8.1 ± 3.4	7.6 ± 6.1
22:5n-3-d_4	1.2 ± 0.2	1.1 ± 0.3
22:5n-3-d_6	0.9 ± 0.3	0.8 ± 0.4
22:6n-3-d_4	0.9 ± 0.8	0.8 ± 0.5
22:6n-3-d_5	2.8 ± 0.7	2.6 ± 0.2
24:6n-3-d_4	0.04 ± 0.02	0.04 ± 0.1
24:6n-3-d_5	0.01 ± 0.01	0.01 ± 0.01

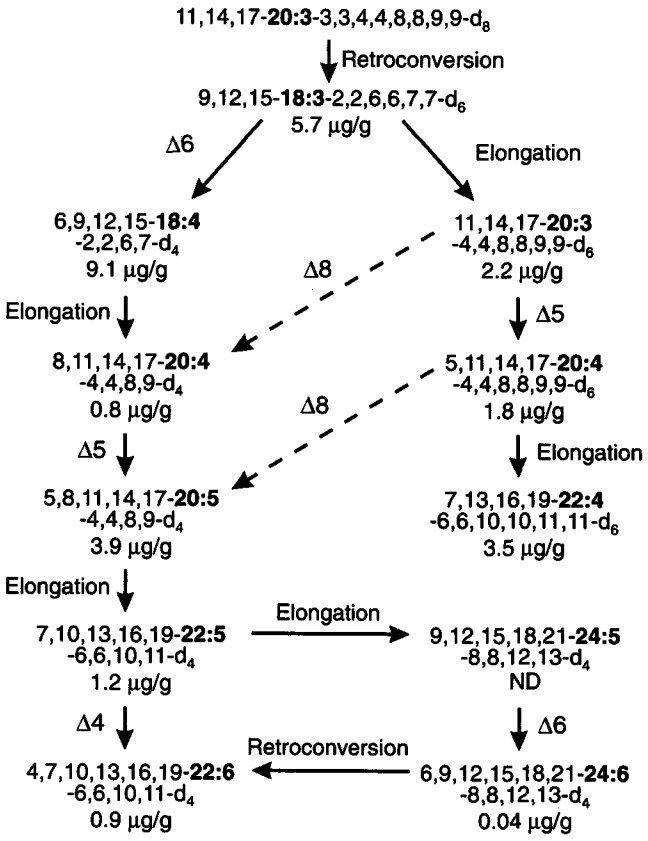
[a]Mean μg/g liver ± SEM; $n = 4$.
[b]Mean % total deuterium ± SEM; $n = 4$.

ing of 20:3n-3-d_8 resulted in the formation of 20:4n-3-d_6, which can only arise *via* a Δ8 desaturation, with the loss of two deuterium atoms. This metabolite can then undergo Δ5 desaturation, producing 20:5n-3-d_6. The 20:5n-3-d_6 metabolite also can be produced *via* the Δ5 desaturation of 20:3n-3-d_8 forming 5*c*20:4n-3-d_8, which can undergo a Δ8 desaturation, resulting in the loss of two deuterium atoms. A 2-carbon chain elongation, Δ4 desaturation, and another 2-carbon chain elongation would result in the production of 22:5n-3-d_6, 22:6n-3-d_5, and 24:6n-3-d_5, respectively. Alternately, 22:5n-3-d_6 may undergo a 2-carbon elongation, forming 24:5n-3-d_6, and then this product may undergo Δ6 desaturation and retroconversion producing 22:6n-3-d_5. Those products that could only be formed as a result of a Δ8 desaturation include 20:4n-3-d_6, 20:5n-3-d_6, 22:5n-3-d_6, 22:6n-3-d_5, and 24:6n-3-d_5, and accounted for 4.4% of the total deuterated products.

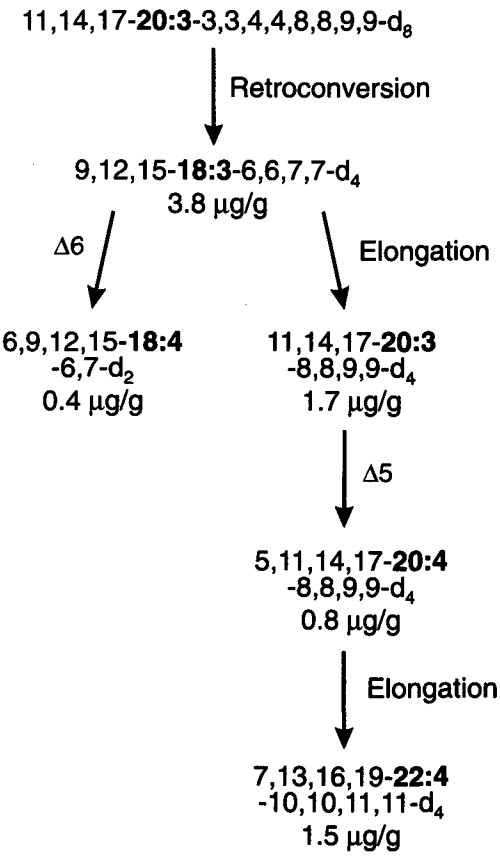
A summary of the deuterated metabolites of 20:3n-3-d_8 is presented in Table 3. Deuterated fatty acids resulting from Δ8 desaturation, retroconversion, and classical pathway accounted for 4.4, 35.3, and 28.9 of the total deuterated fatty acids, respectively. The sum of these products accounts for 68.6% of total deuterated fatty acids isolated. The fed fatty acid 20:3n-3-d_8 comprised 22.3% of the total deuterated fatty acids, and 18:4n-3-d_6, the retroconversion product of 20:4n-3-d_6 and/or -d_8, were responsible for the remaining 9.0% of the total deuterated fatty acids. By comparing the percentage of products resulting from each pathway to the total percentage



SCHEME 1



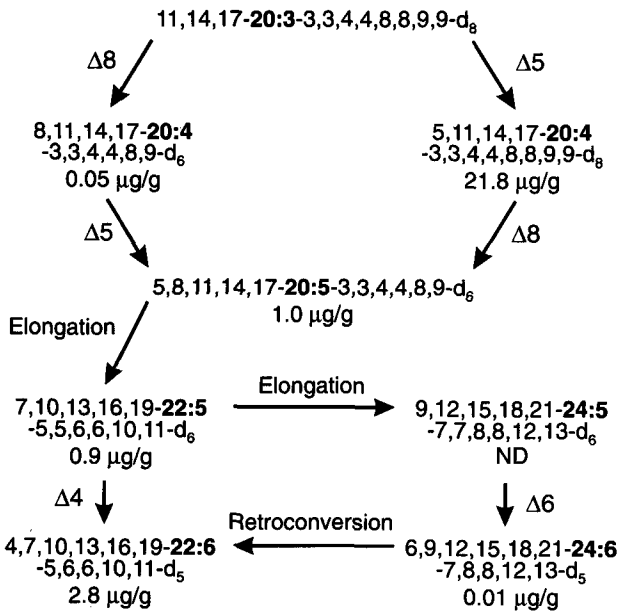
SCHEME 2



SCHEME 3

of products from all three pathways, an approximation of the contribution of each metabolic pathway can be made. Using this approach, 42.1% of metabolites resulted from the classical pathway, 51.5% arose from metabolites involving initial retroconversion, and 6.4% of deuterated metabolites arose only from Δ8 desaturation. However, the percentage contribution of Δ8 desaturation is underestimated in this manner because some of the 5*c*20:4n-3-d_8 formed in the classical pathway also may have been converted to 20:5n-3-d_6 *via* Δ8 desaturation. The reaction sequence of 20:3n-3-d_8 → 18:3n-3-d_6 → 20:3n-3-d_6 would yield 20:4n-3-d_4 *via* Δ8 desaturation of 20:3n-3-d_6. By a similar sequence, 20:5n-3-d_4 could be formed from 5*c*20:4n-3-d_6 (Scheme 2, dashed lines). By feeding 11,14,17-20:3-3,3,4,4,8,8,9,9-d_8, the percentages of 20:4n-3-d_4 and 20:5n-3-d_4 that were formed by Δ8 desaturation cannot be determined.

The presence of Δ8 desaturation activity has been shown in cultured rat glioma cells (9,10), rat testes (7), mouse and human neuroblastoma cells (10), human fibroblast cells (10), human testes (8), and normal and cancerous ovarian, colonic, and urinary bladder tissues (11). However, *in vivo* studies have denied the possibility that Δ8 desaturation occurs in the rat liver (5,6) or in the brain (21,22) which is inconsistent with the presence of small amounts of deuterium labeled Δ8 desaturation products in mouse liver lipids (Table 2). An explanation for the difference between these results is that larger amounts of the deuterium labeled substrate were fed for a much longer time in this study compared to previous studies (3 d vs. 6–8 h). This difference in experimental design could have resulted in the accumulation of detectable amounts of deuterium labeled Δ8 desaturation products whereas the levels of radioactive Δ8 desaturation metabolites



SCHEME 4

TABLE 3
Summary of Deuterated Metabolites of 20:3n-3-3,3,4,4,8,8,9,9-d_8 in Mouse Liver

	μg/g[a]	%TD[b]	%Metab[c]
Sum Δ8 desaturation products (Scheme 4) ($20:5d_6$, $22:5d_6$, $22:6d_5$, $24:6d_5$)	4.76	4.36	6.36
Sum retroconversion products (Schemes 2, 3)	37.34	35.34	51.52
Sum classical products (Scheme 1)	30.60	28.90	42.13
Total	72.70	68.60[d]	100.01

[a]Deuterated fatty acids in μg per gram of liver tissue.
[b]Percentage of total deuterated fatty acids.
[c]Percentage of pathway metabolites derived from Δ8 desaturation, retroconversion, or the classical pathway.
[d]Percentage of total deuterated fatty acids derived from the classical pathway, retroconversion, and Δ8 desaturation.

formed in previous *in vivo* studies were below detection limits.

The present study provides the first clear evidence for Δ8 desaturation *in vivo* in the mouse liver. The overall contribution of Δ8 desaturation to metabolism of fatty acids in the normal mouse remains unclear. In this study, mice were marginally EFA-deficient as evidenced by an accumulation of 1.1% 20:3n-9 in the liver lipids (23). This EFA deficiency can enhance desaturase activities (19), but it is unknown how each specific desaturase is affected. It is possible that Δ8 desaturation is depressed in healthy animals, and only becomes active when the primary Δ6 desaturase pathway is disrupted or inhibited. An inhibition of Δ6 desaturase activity may allow alternative reactions to be enhanced. K562 human leukemia cells, which lack Δ6 desaturase activity, were still able to produce 22:5n-3 and 22:6n-3 from linolenic acid (13). These results are consistent with the suggestion of a Δ8 desaturase as an alternative pathway.

The placement of the deuterium label at the 8,8,9,9 positions in the fed 20:3n-3-d_8 may have played a role in the detection of Δ8 desaturation in this *in vivo* study. The feeding of 11,14,17-20:3-3,3,4,4,8,8,9,9-d_8 resulted in the production of 9,12,15-18:3-2,2,6,6,7,7-d_6 (5.4% of total deuterated fatty acids). This fatty acid would undergo Δ6 desaturation, but the presence of deuterium at the 6 and 7 carbon positions may have reduced the rate of Δ6 desaturation due to a deuterium isotope effect, such as observed for Δ9 desaturation (24). If Δ6 desaturation was reduced, Δ8 desaturation, elongation, and chain-shortening pathways that would not experience a deuterium isotope effect may have been favored. The determination of the magnitude of the deuterium isotope effect on Δ6 desaturase activity is currently under investigation.

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Effects of β-Lactam Antibiotics on Intestinal Microflora and Bile Acid Metabolism in Rats

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ABSTRACT: Wistar male rats were treated for six days with broad spectrum β-lactam antibiotics, latamoxef, and cefotaxime. On the seventh day, the number of fecal anaerobic microbes decreased, total fecal bile acids decreased, and bile acid pools increased. Secondary bile acids such as β-hyocholic, hyodeoxycholic, lithocholic, and deoxycholic acids decreased in the feces while the primary bile acids, cholic, β-muricholic, and chenodeoxycholic acids, became predominant. Coprostanol, a microbial metabolite of cholesterol, also disappeared from the feces during the treatment. The cecum enlarged to almost twice the size of that in control rats, whereas the liver weight was not significantly changed. After treatment was stopped, the number of fecal microbes returned to the initial counts within a week, but restoration of bile acid and cholesterol metabolism required at least three weeks.

Lipids 31, 601–609 (1996).

Intestinal bacteria can transform bile acids and sterols (1–3). Typical reactions are deconjugation, dehydroxylation, oxidation–reduction, and epimerization of bile acids, and also the reduction of cholesterol to coprostanol. Therefore, changes in the intestinal microflora can affect the metabolism of bile acids and sterols. When the numbers of intestinal bacteria were decreased by antibiotics, the fecal bile acid and sterol profiles resembled those under germ-free conditions (4–6). Lindstedt and Norman (4) have reported that treating rats with phthalylsulfatiazole and terramycin affected intestinal microflora and prolonged the elimination rate of cholic acid. Gustafsson *et al.* (5) have examined the effects of neomycin, kanamycin, benzylpenicillin, erythromycin, and bacitracin and showed that the germ-free profile of bile acids lasted for a long time after treatment was stopped. Furthermore, Madsen *et al.* (6) have studied the influence of aureomycin on bile acid metabolism and reported that the hyodeoxycholic acid/β-hyocholic acid (previously named ω-muricholic acid) ratio decreased significantly. These studies showed that antibiotics affected bile acid metabolism, but the relationships among the changes in intestinal microflora and bile acids with cholesterol metabolism remained unknown. Latamoxef (LMOX) and cefotaxime (CFTX) have been recognized to be effective antibiotics and have been widely used for some years (7). The antibacterial spectra of these injectable antibiotics were broader than those of oral cefem antibiotics. After injection of these broad spectrum cefem antibiotics, a part of them was excreted into bile (8–10), and therefore affected the intestinal flora (11). The changes in the flora were expected to affect bile acid metabolism, causing diarrhea or changes in lipid metabolism. In this study, we investigated the changes in bile acid and cholesterol metabolism after treatment with LMOX and CFTX in relation to changes in intestinal microflora in rats.

MATERIALS AND METHODS

Animals. Seven-week-old male Wistar rats were obtained from Aburahi Laboratories, Shionogi (Shiga, Japan) and maintained in an air-conditioned room (25 ± 1°C, 50–60% humidity) under a 12-h photoperiod (8:00–20:00). The rats were caged individually and given a commercially available diet (CA-1, Japan CLEA, Tokyo, Japan) and tap-water *ad libitum.* At the start of the study, the eight-week-old rats were divided into three groups of eight, that were administered saline, LMOX, and CFTX.

Reagents. LMOX was obtained from Shionogi & Co., Ltd. (Osaka, Japan), and CFTX was purchased from Hoechst Japan (Kobe, Japan). Piperidinohydroxypropyl Sephadex LH-20 (PHP LH-20) was obtained from Shimadzu Co., Ltd. (Kyoto, Japan). Other reagents were of analytical grade.

Antibiotic administration and sample collection. LMOX and CFTX were dissolved in sterilized water to make a 25% solution. At 9:00–10:00 a.m., the antibiotics were injected into the rats *via* the tail vein, at a dose of 300 mg/kg/d, once a day for six days. The control rats were given saline *via* the tail vein (1.2 mL/kg/d). At 24 h after the final administration, the bile duct was cannulated with polyethylene PE10 tubing (Clay Adams, Parsippany, NJ), and bile was collected from three rats of each group for 30 min under sodium pentobarbital (65 mg/kg, i.p.) anesthesia. The liver was then removed from each rat, blotted on paper, and weighed. The small and large intestines were removed with their contents. Similarly, at the end of the study (on day 49), bile was collected, and the liver and intestines were removed from five rats in each

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Abbreviations: CFTX, cefotaxime; LMOX, latamoxef.

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group. Feces were collected for two days, dried by lyophilization and powdered in a coffee mill as described previously (12).

Fecal sterol and bile acid determination. Bile acids and sterols were extracted from the powdered feces and determined by gas–liquid chromatography essentially according to Uchida *et al.* (12,13) and Chikai *et al.* (14). The alcoholic extracts of bile acids from the feces on day 7 were separated into conjugated and unconjugated bile acids using a PHP LH-20 column (200 mg per extract from 1 g of dried feces powder) according to Goto *et al.* (15) before alkaline hydrolysis.

Biliary lipid determination. Biliary cholesterol, phospholipids, and bile acids were determined using 0.5 mL of bile by the methods described by Uchida *et al.* (12,13) and Chikai *et al.* (14).

Determination of intestinal bile acids. The small and large intestines with their contents were homogenized and lyophilized. The bile acids were extracted and determined as reported (13).

Determination of intestinal microflora. The fecal contents in the rectum were collected from the anus of three rats in each group, placed in reduced transportation medium (16), and kept at 4°C for 15 h until processed. The method used for analyzing the fecal microflora (17) was essentially the same as described by Mitsuoka *et al.* (18). Briefly, feces was suspended in an anaerobic diluent (19) and serial 10-fold dilutions from 10^{-1} to 10^{-8} were prepared. These dilutions were cultured in 0.05 mL vol onto the surfaces of four nonselective media and 11 selective media (20–22). The dilutions were also heated at 80°C water bath for 10 min to detect clostridial spores. The heated dilutions were cultured on the surfaces of Eggerth-Gagnon (22), glucose blood liver (22), and 5% horse blood Trypticase Soy blood (BBL Microbiology Systems, Cockeysville, MD) agar plates and neomycin-Nagler (22) agar. For the bacterial groups identified, the bacterial count per one g wet feces was calculated and converted into a logarithmic equivalent. The total viable count was calculated from the sum of the counts of each bacterial species. The minimum number detected was 10^2/g wet feces.

RESULTS

Diet intake, body weight gain, and feces dry weight. The diet intake decreased during treatment with the antibiotics, but it was restored to the control level after cessation of the treatment (Fig. 1A). The body weights of the rats given LMOX or CFTX decreased, but then increased after the treatment was stopped (Fig. 1B), in parallel to that in the control rats, but did not exceed the latter throughout the experimental period. The feces dry weight decreased on day 2 but remained almost constant thereafter (Fig. 1C). The rats treated with antibiotics excreted diarrhetic, or soft and wet feces until day 14. The feces were round and larger than those of the control rats. However, the feces excreted on and after day 21 were as hard, dry, and small as those of the control rats.

Weight of the liver and large intestine. The data are shown in Table 1. The liver weight decreased in the rats treated with

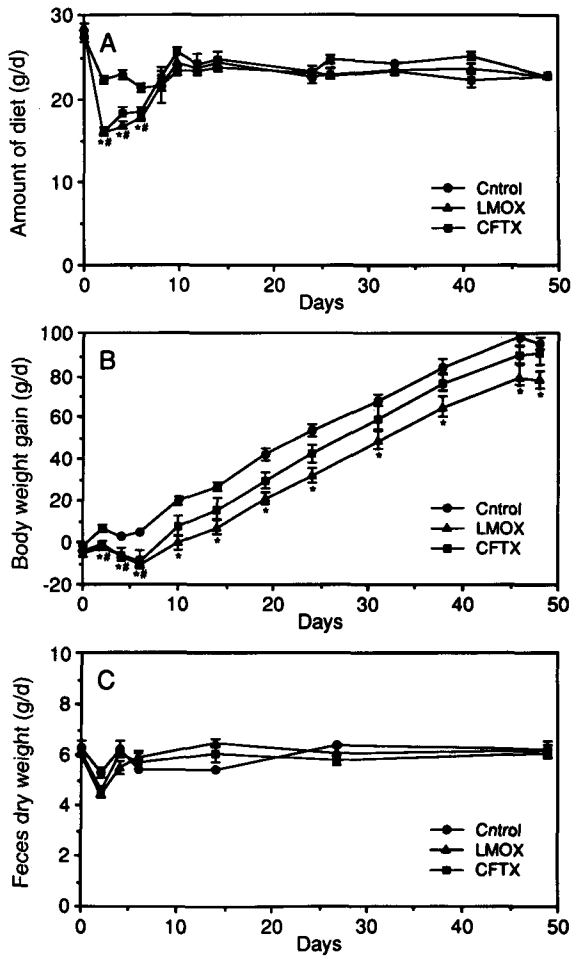


FIG. 1. Changes in diet intake, body weight gain, and feces dry weight in control and antibiotic-treated rats. Both antibiotics, latamoxef (LMOX) and cefotaxime (CFTX), were given intravenously (i.v.) once a day for six days (day 1 through day 6).

LMOX on day 7, but no difference was found on day 49. CFTX tended to reduce the liver weight on day 7, but the change was statistically insignificant. The cecum was markedly dilated by the antibiotics. Therefore, the large intestine weights markedly increased on day 7, but were restored to the control value on day 49.

Intestinal microflora. The intestinal microflora consisted mainly of eight bacterial groups before the course of antibiotics (Table 2). LMOX caused most of them to disappear, thus decreasing the total count, but the levels of *Streptococci* remained unchanged and a few *Lactobacilli* were found in one rat. However, these bacteria recovered to the original counts one week after cessation of the treatment (on day 14) and remained unchanged thereafter. In the CFTX-treated rats, *Enterobacteriaceae* were abolished, and the levels of *Bacteroidaceae* and *Micrococci* were partially decreased, but all reappeared after treatment was stopped.

Fecal bile acids and sterols. The fecal excretion of bile acids and sterols in the control and the antibiotic treated rats

TABLE 1
Effects of Antibiotics on Liver and Large Intestine Weights in Rats

	Day 7			Day 49		
	Control	LMOX[a]	CFTX[a]	Control	LMOX[a]	CFTX[a]
Number of rats	3	3	3	5	5	5
Liver weight (g/100 g body weight)	3.45 ± 0.04[b]	3.15 ± 0.05[c]	3.25 ± 0.08	3.16 ± 0.07	3.23 ± 0.02	3.25 ± 0.06
Large intestine weight (g)	7.64 ± 0.21	16.18 ± 0.64[c]	12.89 ± 0.15[c]	7.42 ± 0.19	7.42 ± 0.82	8.31 ± 0.56

[a]Antibiotics (300 mg/d/rat) were injected intravenously for six days (day 1 through day 6), and the animals were sacrificed on day 7 and day 49. LMOX, latamoxef; CFTX, cefotaxime.
[b]Mean ± SE.
[c]Statistically significant compared to the paired controls ($P < 0.05$).

TABLE 2
Effects of Antibiotics on Intestinal Microflora in Rats

	LMOX[a]				CFTX[a]			
	Day 0	Day 7	Day 14	Day 27	Day 0	Day 7	Day 14	Day 27
Total count	10.5[b]	6.7	10.6	10.3	10.6	9.9	10.2	10.1
Bacteroidaceae	10.1		9.8	9.9	10.1	8.6 (1/3)	9.7	9.4
Eubacterium			9.0 (1/3)		9.5 (1/3)		9.0 (1/3)	
Peptococcaceae	9.9 (2/3)		9.2	9.2 (1/3)			8.2	
Bifidobacterium						8.0 (2/3)		
Clostridium-others	7.9		10.0	8.4	8.0 (1/3)	7.9	9.0	7.0
Lactobacillus	10.3	2.2 (1/3)	10.0	9.8	10.3	8.9	9.8	10.0
Enterobacteriaceae	7.6		5.8	7.4	7.8		5.3	6.9
Streptococcaceae	6.8	6.7	7.3	7.2	7.2	9.6	7.7	7.3
Micrococcaceae	5.6		4.1	4.1	5.0	2.6 (1/3)	6.0	4.6
Corynebacterium	5.9							
Bacillus				3.9 (1/3)				2.9 (2/3)

[a]Antibiotics (300 mg/d/rat) were injected intravenously for six days (day 1 through day 6). See Table 1 for abbreviations.
[b]Values are Log number of bacteria per gram wet weight of feces. Numbers of rats are three and values in parentheses indicate number of rats which had a single type of bacterium.

on day 7 and day 49 is shown in Table 3. The total fecal bile acids in the LMOX- or CFTX-treated rats markedly decreased, to less than 30% of the controls, but were restored to the control level on day 49. The levels of the secondary bile acids, such as deoxycholic, lithocholic, β-hyocholic, and hyodeoxycholic acids, decreased and the percentages of the primary bile acids, such as cholic, chenodeoxycholic, and β-muricholic acids, increased on day 7. Similarly, the total fecal sterols in the treated rats decreased on day 7 but were restored on day 49. The cholesterol levels in the treated rats on day 7 were comparable with that in the controls, but coprostanol was almost completely diminished in the treated rats.

The bile acid levels periodically examined in the feces are shown in Figure 2. The control levels, though they fluctuated somewhat, were almost constant throughout the experimental period, with values of 7–8 mg/d/rat. The levels in the treated rats decreased after one week but were similar to those in the controls in the other determination days. The primary bile acids which comprised cholic, chenodeoxycholic, α-muricholic, and β-muricholic acids increased after one and two weeks, while the secondary bile acids decreased on day 7 but were almost completely restored to the control levels after four weeks or later.

Changes in the individual fecal bile acids are shown in Figure 3 in three groups, since cholic acid is considered to be converted by the intestinal bacteria to 7-oxo-deoxycholic and 7β-cholic acids, β-muricholic acid to β-hyocholic acid then to hyodeoxycholic acid, and chenodeoxycholic acid to lithocholic acid. The percentages of the individual bile acids were quite constant in the control rats, but deoxycholic acid decreased with a concomitant increase in cholic acid after one week, β-hyocholic acid plus hyodeoxycholic acid decreased with an increase in β-muricholic acid after one and two weeks, and lithocholic acid decreased with an increase in chenodeoxycholic acid after one week in both LMOX- and CFTX-treated rats.

The changes in the fecal sterols are shown in Figure 4. Coprostanol almost completely disappeared after one week but reappeared after two weeks or later. The fecal excretion of cholesterol after two weeks in the treated rats was significantly higher than that in the controls.

Most of the fecal bile acids in control rats were deconjugated, but they were excreted into feces as conjugates after treatment with the antibiotics. As shown in Table 4, about 94% of the fecal bile acids in the LMOX-treated rats were conjugated with taurine. This value was lower (70%) in the CFTX-treated rats. However, β-muricholic acid was 100%

TABLE 3
Effects of Antibiotics on Fecal Bile Acids and Sterols in Rats

	Day 7			Day 49		
	Control	LMOX[a]	CFTX[a]	Control	LMOX[a]	CFTX[a]
Number of rats	8	8	8	5	5	5
Total bile acids (mg/d/rat)	7.45 ± 09.76[b]	2.16 ± 0.18[c]	2.05 ± 0.26[c]	6.94 ± 0.84	6.68 ± 0.49	8.04 ± 1.21
Cholic acid group (mg/d/rat)	2.43 ± 0.31	1.23 ± 0.12[c]	1.15 ± 0.14[c]	2.07 ± 0.12	1.89 ± 0.23	1.82 ± 0.12
Cholic acid (%)	1 ± 0.2	35 ± 1.4[c]	33 ± 5.6[c]	trace	trace	trace
Deoxycholic acid (%)	16 ± 0.8	trace	trace	25 ± 2.8	22 ± 1.4	21 ± 2.5
7β-Cholic acid (%)	2 ± 0.3	trace	trace	trace	2 ± 0.4	trace
7-Oxo-deoxycholic acid (%)	5 ± 0.7	trace	4 ± 1.9	4 ± 0.7	5 ± 1.0	3 ± 2.1
Chenodeoxycholic acid group (mg/d/rat)	5.02 ± 0.49	0.92 ± 0.07[c]	0.90 ± 0.19[c]	4.88 ± 0.77	4.79 ± 0.40	6.22 ± 1.22
Chenodeoxycholic acid (%)	5 ± 0.6	12 ± 1.1[c]	14 ± 3.2[c]	7 ± 0.9	7 ± 1.2	2 ± 1.2[c]
α-Muricholic acid (%)	1 ± 0.1	3 ± 0.8	3 ± 0.6	2 ± 0.2	1 ± 0.3	1 ± 0.2
β-Muricholic acid (%)	13 ± 2.3	21 ± 0.7[c]	14 ± 1.0	5 ± 1.1	5 ± 1.7	3 ± 1.1
β-Hyocholic acid (%)	32 ± 4.1	6 ± 0.2[c]	11 ± 4.7[c]	32 ± 5.2	26 ± 6.6	12 ± 2.0[c]
Lithocholic acid (%)	7 ± 0.4	trace	trace	8 ± 0.6	8 ± 0.4	7 ± 0.8
Hyodeoxycholic acid (%)	6 ± 6.2	trace	trace	7 ± 4.5	18 ± 11.0	43 ± 10.3
β-Hyocholic acid + Hyodeoxycholic acid (%)	37 ± 3.2	6 ± 0.2[c]	11 ± 4.7[c]	39 ± 4.4	44 ± 6.5	54 ± 8.4
Total sterols (mg/d/rat)	17.6 ± 0.8	11.7 ± 0.6[c]	11.8 ± 0.7[c]	22.3 ± 1.2	21.1 ± 0.3	22.2 ± 0.4
Cholesterol (mg/d/rat)	10.5 ± 1.0	11.7 ± 0.6	11.8 ± 0.7	11.4 ± 0.5	10.0 ± 0.8	10.5 ± 0.6
Coprostanol (mg/d/rat)	7.1 ± 0.9	trace	trace	10.9 ± 1.1	11.2 ± 0.9	11.7 ± 0.4

[a]Antibiotics (300 mg/d/rat) were injected intravenously for six days (day 1 through day 6), and the animals were sacrificed on day 7 and day 49. See Table 1 for abbreviations.
[b]Mean ± SE.
[c]Statistically significant compared to the paired controls ($P < 0.05$).

conjugated with taurine in both groups. Cholic acid was 93 and 53% conjugated in the LMOX- and CFTX-treated rats, respectively. Neither glycine conjugates nor sulfates were detected.

Bile flow and biliary lipid concentrations. The bile flow and biliary lipid concentrations are given in Table 5. The bile flow of the treated rats was lower than that of the control rats, but this difference was statistically insignificant. The biliary cholesterol and phospholipid concentrations were not changed, but the bile acid concentration increased in the treated rats, and the increases were sustained until day 49.

Biliary bile acid composition. The changes in the biliary bile acid composition are given in Table 6. Rat biliary bile acids consisted of two major primary bile acids, cholic and β-muricholic acids, as well as minor primary and secondary bile acids. The percentages of cholic acid were similar in all the groups, while those of β-muricholic acid were increased by the antibiotics (day 7) but rather decreased after treatment was stopped (day 49).

Pool size of bile acids. The pool size of bile acids was calculated by totaling the amounts in the bile (collected for 30 min) and the small and large intestines (Table 7). The pool size was increased by the antibiotics (day 7), but returned to the control level after treatment was stopped (day 49). The percentages of cholic acid were similar in all the groups, but those of β-muricholic acid increased on day 7 in the antibiotic-treated groups. The percentages of the secondary bile acids, though they were minor, decreased on day 7 but returned to the control values on day 49.

The small intestine contained most of bile acids in the pool, amounting to around 80%. The percentages in the large intestine on day 7 were lower in the treated rats than in the controls, though the weight of the large intestine increased by almost twice in the treated rats (Table 1).

DISCUSSION

The antibiotics, LMOX and CFTX, decreased intestinal bacteria, increased the cecal weight, and caused changes in bile acid and cholesterol metabolism in conventional male rats. In the LMOX-treated rats, most of the intestinal bacteria disap-

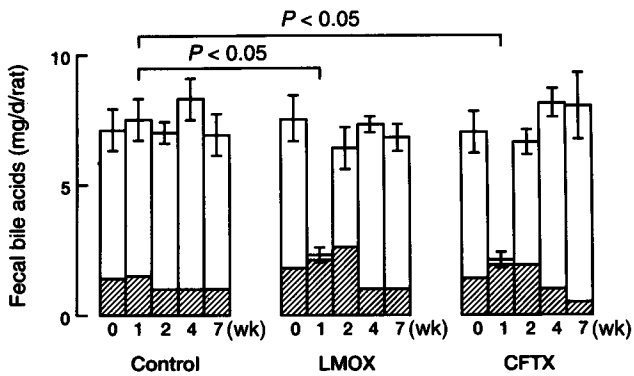


FIG. 2. Changes in fecal bile acids in control and antibiotic-treated rats. The columns and bars show the mean values and standard errors. Shaded columns correspond to the mean value of the primary bile acids. Both antibiotics, LMOX and CFTX, were given intravenously once a day for six days (day 1 through day 6). See Figure 1 for abbreviations.

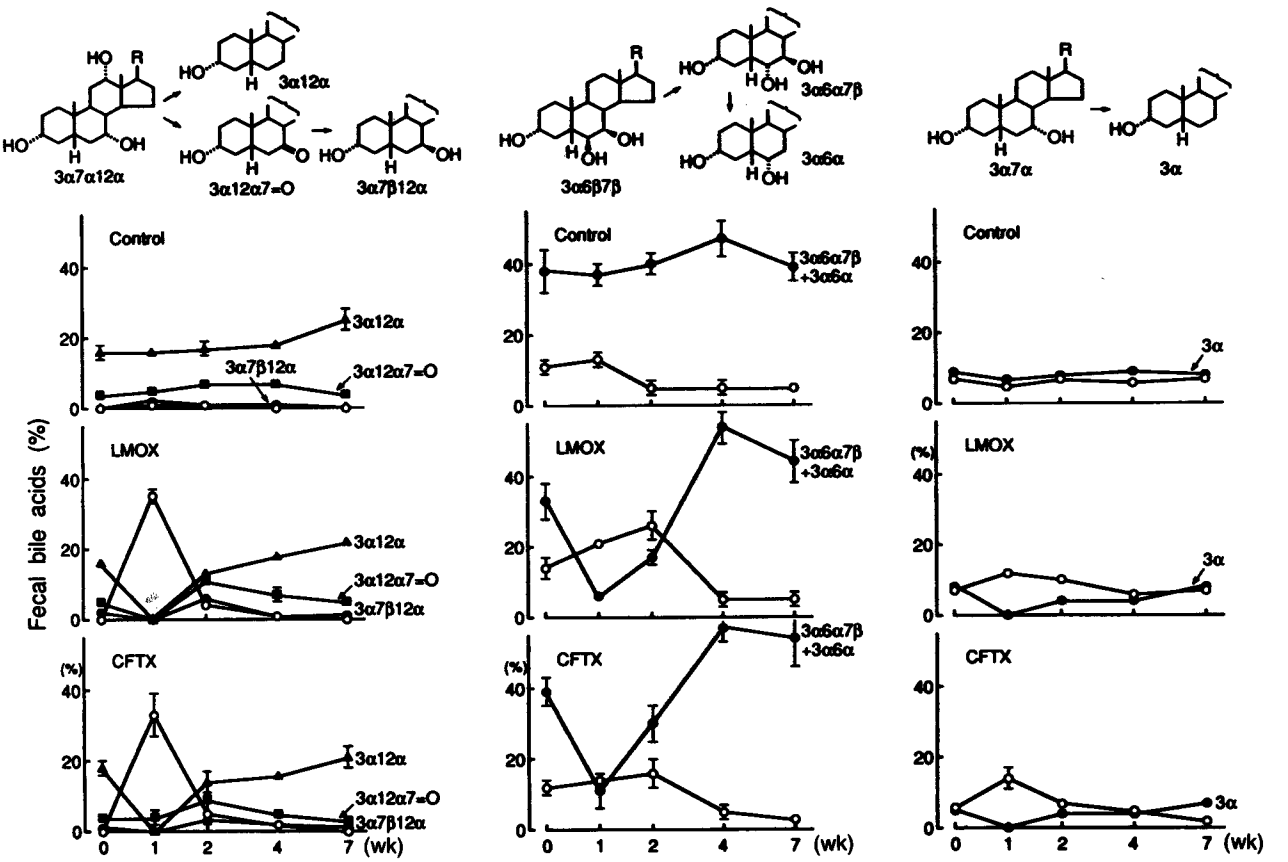


FIG. 3. Changes in fecal bile acid composition in control and antibiotic-treated rats. Both antibiotics, LMOX and CFTX, were given intravenously once a day for six days (day 1 through day 6). The left part shows the changes in cholic acid, the middle in β-muricholic acid, and the right in chenodeoxycholic acid. These primary bile acids are shown as open circles. See Figure 1 for abbreviations.

peared, but *Streptococci* survived in all of them and a few *Lactobacilli* remained in one out of three.

These data were similar to the findings of Benno *et al.* (11), who examined the effect of LMOX on fecal bacteria in humans and reported a significant decrease in most of the anaerobes, but one species of *Clostridium* (*C. innocum*), as well as *Lactobacillus*, *Streptococcus*, and *Candida* survived. In the CFTX-treated rats, *Enterobacteriacea* disappeared but *Clostridium*-others (*Clostridium* species other than *C. difficile*), *Lactobacillus*, and *Streptococcaceae* remained. The difference in surviving microflora between LMOX and CFTX was partially due to the biliary excretion of the antibiotics. The biliary excretion of LMOX is about 20% after intravenous injection (8), but that of CFTX is maximally 8% in rats (9,10). The fecal microflora, however, returned to the initial counts one week after cessation of the treatment in both groups.

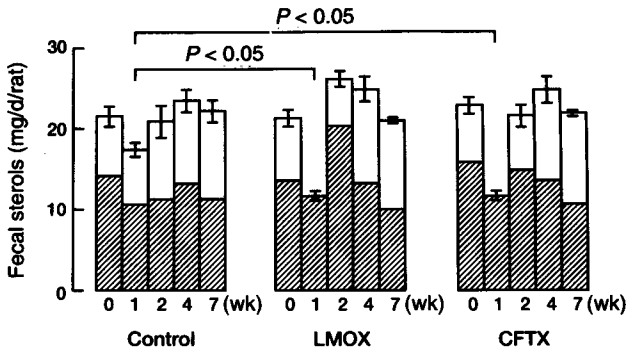


FIG. 4. Changes in fecal sterols in control and antibiotic-treated rats. The columns and bars show the mean values and standard errors. Shaded columns correspond to the mean values of cholesterol. Both antibiotics, LMOX and CFTX, were given intravenously once a day for six days (day 1 through day 6). See Figure 1 for abbreviations.

TABLE 4
Percentages of Taurine-Conjugated Bile Acids in Feces

	Control	LMOX[a]	CFTX[a]
Number of rats	8	8	8
Total	trace	94 ± 1.3[b]	70 ± 6.8[c]
Cholic acid	trace	93 ± 1.9	53 ± 5.3[c]
Chenodeoxycholic acid	trace	68 ± 5.2	63 ± 3.2
α-Muricholic acid	trace	96 ± 2.9	82 ± 3.9[c]
β-Muricholic acid	trace	100	100

[a]Antibiotics (300 mg/d/rat) were injected intravenously for six days (day 1 through day 6), and the feces were analyzed on day 7. See Table 1 for abbreviations.
[b]Mean ± SE.
[c]Statistically significant compared to the LMOX-treated rats ($P < 0.05$).

TABLE 5
Effects of Antibiotics on Bile Flow and Biliary Lipid Concentrations in Rats

	Day 7			Day 49		
	Control	LMOX[a]	CFTX[a]	Control	LMOX[a]	CFTX[a]
Number of rats	3	3	3	5	5	5
Bile flow (mL/h)	1.29 ± 0.06[b]	1.16 ± 0.08	1.08 ± 0.03	1.42 ± 0.07	1.26 ± 0.03	1.29 ± 0.04
Cholesterol (mg/mL)	0.12 ± 0.02	0.12 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.10 ± 0.00
Phospholipid (mg/mL)	3.33 ± 0.15	3.74 ± 0.21	3.23 ± 0.14	3.19 ± 0.32	3.44 ± 0.31	2.67 ± 0.10
Bile acids (mg/mL)	8.84 ± 2.72	19.30 ± 2.42[c]	18.43 ± 0.93[c]	10.15 ± 1.22	15.05 ± 1.68[c]	14.91 ± 1.40[c]

[a]Antibiotics (300 mg/d/rat) were injected intravenously for six days (day 1 through day 6), and the animals were sacrificed on day 7 and day 49. See Table 1 for abbreviations.
[b]Mean ± SE.
[c]Statistically significant compared to the paired controls ($P < 0.05$).

TABLE 6
Effects of Antibiotics on Biliary Bile Acid Composition in Rats

	Day 7			Day 49		
	Control	LMOX[a]	CFTX[a]	Control	LMOX[a]	CFTX[a]
Number of rats	3	3	3	5	5	5
Cholic acid group						
Cholic acid (%)	48 ± 1.3[b]	42 ± 2.6	46 ± 0.4	46 ± 3.3	47 ± 1.8	47 ± 1.9
Deoxycholic acid (%)	4 ± 0.8	trace	trace	3 ± 0.4	4 ± 0.4	4 ± 0.1
7β-Cholic acid (%)	trace	trace	trace	trace	trace	1 ± 0.3
7-Oxo-deoxycholic acid (%)	2 ± 0.3	trace	trace	2 ± 0.3	2 ± 0.2	2 ± 0.5
Chenodeoxycholic acid group						
Chenodeoxycholic acid (%)	8 ± 1.3	5 ± 1.2	5 ± 0.1	5 ± 0.7	9 ± 1.4	5 ± 0.1
α-Muricholic acid (%)	4 ± 0.8	1 ± 0.1[c]	1 ± 0.1[c]	1 ± 0.2	1 ± 0.2	trace
β-Muricholic acid (%)	32 ± 2.9	47 ± 1.4[c]	41 ± 1.3[c]	34 ± 5.6	21 ± 2.1	21 ± 1.6
β-Hyocholic acid (%)	2 ± 0.3	trace	trace	3 ± 0.3	2 ± 0.2	2 ± 0.1
Lithocholic acid (%)	trace	trace	trace	trace	4 ± 0.9	3 ± 0.7
Hyodeoxycholic acid (%)	trace	trace	trace	trace	2 ± 1.1	6 ± 1.2[c]

[a]Antibiotics (300 mg/d/rat) were injected intravenously for six days (day 1 through day 6), and the animals were sacrificed on day 7 and day 49. See Table 1 for abbreviations.
[b]Mean ± SE.
[c]Statistically significant compared to the paired controls ($P < 0.05$).

TABLE 7
Effects of Antibiotics on Pool Size and Distribution of Bile Acids in Rats

	Day 7			Day 49		
	Control	LMOX[a]	CFTX[a]	Control	LMOX[a]	CFTX[a]
Number of rats	3	3	3	5	5	5
Pool size (mg/rat)	45.4 ± 0.98[b]	58.6 ± 2.16[c]	63.8 ± 1.28[c]	47.1 ± 4.39	43.1 ± 4.08	43.7 ± 5.80
Cholic acid group						
Cholic acid (%)	47 ± 2.3	48 ± 0.4	49 ± 1.3	48 ± 2.4	51 ± 2.6	49 ± 3.2
Deoxycholic acid (%)	3 ± 0.0	trace	trace	3 ± 0.2	3 ± 0.4	4 ± 0.6
7β-Cholic acid (%)	trace	trace	trace	3 ± 2.3	1 ± 0.5	trace
7-Oxo-deoxycholic acid (%)	2 ± 0.1	1 ± 0.1	1 ± 0.1	2 ± 1.0	3 ± 0.5	3 ± 0.5
Chenodeoxycholic acid group						
Chenodeoxycholic acid (%)	5 ± 0.5	4 ± 0.3	2 ± 1.1	4 ± 0.7	5 ± 0.2	5 ± 0.5
α-Muricholic acid (%)	1 ± 0.1	1 ± 0.1	1 ± 0.0	1 ± 0.2	1 ± 0.2	1 ± 0.3
β-Muricholic acid (%)	37 ± 2.0	44 ± 0.3[c]	44 ± 1.5	26 ± 3.9	23 ± 2.3	23 ± 1.6
β-Hyocholic acid (%)	4 ± 0.4	1 ± 0.1	1 ± 0.1	6 ± 3.6	3 ± 0.2	2 ± 0.2
Lithocholic acid (%)	trace	trace	trace	trace	1 ± 0.1	trace
Hyodeoxycholic acid (%)	trace	trace	trace	2 ± 0.9	4 ± 0.7	8 ± 1.7
Distribution (%)						
Bile	13 ± 3.7	19 ± 3.1	16 ± 0.6	15 ± 1.6	22 ± 1.5c	22 ± 5.5
Small intestine	84 ± 3.4	80 ± 3.2	83 ± 0.8	82 ± 1.8	76 ± 1.6	75 ± 5.4
Large intestine	3 ± 0.3	1 ± 0.1[c]	2 ± 0.3[c]	3 ± 0.5	2 ± 0.4	3 ± 0.2

[a]Antibiotics (300 mg/d/rat) were injected intravenously for six days (day 1 through day 6), and the animals were sacrificed on day 7 and day 49. See Table 1 for abbreviations.
[b]Mean ± SE.
[c]Statistically significant compared to the paired controls ($P < 0.05$).

A pronounced enlargement of the cecum is a general feature in germ-free rodents (23–25). Wostmann *et al.* (25) have suggested that this is due to lack of an enzyme(s) of microbial origin that inhibits the accumulation of high molecular weight and negatively charged mucopolysaccharide(s) in the cecum. In this study, LMOX caused a larger decrease in the fecal bacterial counts and a larger increase in the cecum weight when compared with CFTX.

The diet-intake and body weight gain decreased during the treatment, though the maximal "no effect" dose of LMOX was reported to be 1260 mg/kg/d (i.v.) from a subacute toxicity study in rats (26). Handling of rats sometimes caused decreases in food-intake and body weight gain, but the larger decreases in the antibiotic-treated rats were statistically significant.

During the treatment with the antibiotics, the level of secondary bile acids decreased with a concomitant increase in that of primary bile acids in the feces. Furthermore, most of the primary bile acids in the feces were conjugated with taurine. Glycine-conjugated bile acids, as well as those conjugated with taurine and their sulfates, are found in various animal species, but glycine–conjugates and sulfates are found at low levels in rats (27). Neither glycine–conjugates nor sulfates were detectable in this study. The percentages of taurine–conjugates differed according to individual bile acids and antibiotics, the highest level being with β-muricholic acid and the lowest with chenodeoxycholic and cholic acids in the LMOX- and CFTX-treated rats, respectively. We demonstrated that *Bacteroides vulgatus* preferentially deconjugated tauro-β-muricholic acid and *Bifidobacterium longum* taurocholic acid (14). *Clostridium ramosum* deconjugated both bile acids, but *Escherichia coli* deconjugated neither. Furthermore, we found that *Peptostreptococcus products* and *Lactobacillus gasseri* deconjugated both bile acids in rats, though to a lesser extent than that of *C. ramosum* (28). In this study, the bacteria remaining after the antibiotic administration exerted deconjugation activities to some extents, especially in the presence of CFTX. Cholylglycine hydrolase isolated from *C. perfringence* (29,30) and *B. fragilis* (31) can hydrolyze both taurocholic acid and taurochenodeoxycholic acid, but a hydrolase isolated from *B. vulgatus* (32) preferentially hydrolyzes taurochenodeoxycholic and tauro-β-muricholic acids with far less activity to taurocholic acid. The low percentages of taurochenodeoxycholic acid in the CFTX-treated rats can be explained by the high counts of *Clostridium*-others in this group. On the other hand, *B. vulgatus* possesses high hydrolase activity for tauro-β-muricholic acid (32), but this genus (*Bacteroides*) is sensitive to LMOX and CFTX (Table 2) (11). This would explain the high percentages of tauro-β-muricholic acid in the feces.

A major secondary bile acid from cholic acid is deoxycholic acid, but a small amount of 7β-cholic acid is also formed probably *via* 7-oxo-deoxycholic acid. The latter is also converted to deoxycholic acid *in vivo* (33). LMOX and CFTX decreased these secondary bile acids, while the primary cholic acid concomitantly increased. These changes returned to the initial state three weeks after the treatment was stopped. Chenodeoxycholic acid is a minor primary bile acid in the rat, and its secondary bile acid is lithocholic acid. LMOX and CFTX decreased lithocholic acid and increased chenodeoxycholic acid levels, but they returned to the initial state three weeks after cessation of the treatment. Another major primary bile acid in rats is β-muricholic acid which is formed from chenodeoxycholic acid in the liver (34). Intestinal bacteria convert β-muricholic acid to β-hyocholic acid; Robben *et al.* (35) have proposed that *Clostridium* sp. is responsible not only for this conversion, but also for the further conversion to hyodeoxycholic acid by bacterial 7β-dehydroxylation. Ohta *et al.* (36) have postulated that hyodeoxycholic acid is formed directly from β-muricholic acid by 7β-dehydroxylation *via* 3α,7β-dihydroxychol-6-enoic acid. When hyodeoxycholic acid was given to rats, no significant increase in β-hyocholic acid was found in the feces, but when ursodeoxycholic acid was given, β-hyocholic acid, hyodeoxycholic acid, and also β-muricholic acid levels increased in the feces (37). It is presumed, therefore, that ursodeoxycholic acid is converted to β-muricholic acid in the liver, then to β-hyocholic acid and to hyodeoxycholic acid by intestinal bacteria in the rat. The amounts of β-hyocholic acid plus hyodeoxycholic acid in the feces were quite constant, though they varied individually in inverse proportion (38,39). The sum of β-hyocholic acid and hyodeoxycholic acid was almost constant during the period of experiment in the control rats, but were decreased by LMOX and CFTX, then restored the initial values about three weeks after stop of the treatment.

As long as hepatic and intestinal function are unaltered, the amounts of bile acids excreted into feces are considered to correspond to the amounts of bile acids synthesized in the liver. The fecal excretion of bile acids markedly decreased during the antibiotic treatment and returned to the control level after stopping the treatment. However, since the bile acid pools in the treated rats increased during the period, some of the bile acids, which normally would have been excreted into feces, were stored in the pool. However, the decrease in the fecal bile acids was far larger than the increase in the pool. Therefore, it seems likely that bile acid synthesis was decreased by the antibiotics. In addition, it is possible that antibiotic-induced changes in intestinal function could have resulted in increased resorption of bile acids. Both cholic and chenodeoxycholic acid groups decreased during the treatment, but the decrease in the latter was larger than that in the former. In other words, the synthesis of β-muricholic acid may have decreased more than that of cholic acid, and this could explain why β-muricholic acid in the feces did not increase as much as cholic acid during the antibiotic administration (Fig. 3).

It also was presumed that the decrease in deconjugation and transformation of the primary bile acids increased the reabsorption of bile acids in the intestine, resulting in an increase in the bile acid pool. In other words, the secondary bile acids were less absorbable than the conjugated primary bile acids in the ileum.

Cholesterol is also transformed by intestinal bacteria mainly to coprostanol (3), involving cholest-4-en-3-one and coprostanone (2). Coprostanol comprised about 40% of the total sterols in feces before LMOX and CFTX were administered, but disappeared during the treatment and appeared again thereafter. The percentages of coprostanol in the treated rats were lower than those in the control rats one week after the treatment stopped, but returned to the control level three weeks later. In addition, the amounts of the total sterols in feces were lower during antibiotic administration, probably due to a decrease in the food intake during this period, since the diet contained 0.05–0.1% of cholesterol.

Bile acid metabolism in germ-free rats differs from that in conventional rats (40). Taurine conjugated primary bile acids are detected in germ-free rats, but various bile acids, including the secondary bile acids, are found in conventional rats. The pool size of bile acids is remarkably expanded, the biliary bile acid secretion slightly increased, and the apparent bile acid synthesis based upon reduced fecal bile acid excretion, decreased when compared with the age-matched conventional rats. The relative synthesis of cholic acid is decreased compared to chenodeoxycholic acid (actually β-muricholic acid in the rat), resulting in a decrease in the cholic acid group/chenodeoxycholic acid group ratio in germ-free rats. These changes in germ-free rats seem to resemble those in the present antibiotic-treated rats, except for a remarkable increase in the biliary bile acid secretion in the antibiotic-treated rats.

The evidence described above suggested that treating conventional rats with broad spectrum β-lactam antibiotics brought their bile acid and cholesterol metabolism to the levels of those in a germ-free state. The recovery of fecal microflora population occurred one week after cessation of the treatment, but that of bile acid and cholesterol metabolism required at least three weeks.

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Dietary Fatty Acid Composition Induces Comparable Changes in Cardiolipin Fatty Acid Profile of Heart and Brain Mitochondria

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ABSTRACT: The fatty acid profile of cardiolipin (CL) from brain and cardiac mitochondria was measured to determine whether CL isolated from these two tissue sources responded similarly to alterations in dietary fat composition. Male Wistar rats were fed 20% (w/w) diets containing 2 to 12% (w/w) 18:2n-6 for four weeks. Despite higher baseline levels of CL 18:2n-6 in cardiac (54 ± 1% of total fatty acids) compared to brain (13 ± 1%) mitochondria, CL 18:2n-6 levels increased in proportion to dietary 18:2 levels. The degree of change in 18:2n-6 was comparable with both tissues showing an approximate 1.5- to 2-fold increase. The time course of changes in CL fatty acid profile was examined in a subsequent experiment in which animals were fed 20% (w/w) fat diets containing either 3 or 15% α-linoleate. Changes in cardiac CL 18:1, 18:2n-6, and 22:6n-3 levels were observed within one week of feeding. While statistically significant differences were not observed in brain CL until the second week of feeding, the time course did not differ substantively from that observed in heart. The results from this study suggest that while baseline fatty acid profile of cardiac and neural CL differ, mitochondria from both tissues show comparable sensitivity to changes in dietary fat composition. Furthermore, it would appear that the turnover rate of fatty acids in CL is similar in both tissues.
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Qualitative differences in dietary fat affect the fatty acid composition of most, if not all, membranes in mammalian species. These effects are more evident with essential fatty acid (EFA) deficiency, or during periods of rapid tissue, and hence biomembrane, accretion that characterize fetal and neonatal growth. The degree of sensitivity of membranes appears to differ, with it generally being considered that brain is less susceptible to dietary-induced changes in comparison to peripheral tissues (see for example Refs. 1–4), based originally on the assumption that the blood-brain barrier restricts the passage of fatty acids to brain (5). Nevertheless, dietary fat-induced alterations in brain phospholipid fatty acid profile are observed even in the absence of EFA deficiency when dietary treatment is commenced in post-weanling rats (6–8) and can be accompanied by changes in membrane-bound enzyme activity (9,10). The physiologic relevance of these diet-induced alterations can be attested to by the fact that behavioral changes, including pain sensitivity (11), feeding behavior (6,9), and cognition (12–15), occur in tandem with the biochemical alterations.

Most studies examining the effect of dietary fat on neural membrane fatty acid composition have focused upon differences in the long-chain polyenoic metabolites of linolenate (18:3n-3), namely eicosapentanoate (22:5n-3) and docosahexanoate (22:6n-3) (8,16–18). Recently we (19) described differences in neural α-linoleate (18:2n-6) in response to variable amounts of dietary EFA. The effect was most evident in mitochondrial cardiolipin (CL), and could be observed within one week of feeding the experimental diets. Maximal differences occurred after three weeks of dietary treatment.

Other reports have described the effect of dietary fat on mitochondrial fatty acid composition in peripheral tissues (20–27). However, specific comparisons between brain and peripheral tissues with respect to mitochondrial CL sensitivity to alterations in EFA have not been made. The objective of this study was to compare the effect of dietary 18:2n-6 on mitochondrial CL fatty acid composition in neural and cardiac tissues. We chose to compare the effect of dietary fat on the fatty acid composition of CL because mitochondria from these two tissues differ both from a biochemical and histologic perspective. That is, in comparison to brain mitochondria, mitochondria isolated from heart are more numerous (greater number of mitochondria/cell), have a larger inner membrane leaflet resulting in greater folding of the inner membrane, have higher amounts of CL in the inner leaflet, and have higher concentrations of 18:2n-6 within the CL. These differences in the mitochondria may result in different sensitivities to dietary-induced modification.

EXPERIMENTAL PROCEDURES

Animals and diets. Male Wistar rats (Charles River, St. Constant, Quebec, Canada) were housed individually in galva-

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Abbreviations: ANOVA, analysis of variance; CL, cardiolipin; EFA, essential fatty acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

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nized wire-mesh cages in a temperature-controlled environment (24 ± 1°C) with a 12-h light/dark cycle. Initial body weights were 60–90 g. Animals were weighed at the beginning and the end of the experimental period.

Diets were nutritionally adequate, purified granular mixtures containing 20% fat, 40% carbohydrate, 24% protein, 5% nonnutritive fiber, 2.5% vitamin mix, 5.1% mineral mix, and 0.25% L-methionine. A detailed description of diet ingredients has been published (6). Protein content was verified with the microkjeldahl technique (28). Diets contained vitamin E as an antioxidant (4400 I.U. α-tocopheryl acetate/kg diet). In similar diets, levels of thiobarbituric acid reactive substances ranged from 1.96 to 5.02 μmol/kg diet after 48 h at room temperature (29).

Experiment 1. Rats (n = 10–11/treatment) were randomly assigned to 20% (w/w) fat diets with polyunsaturated fatty acid (PUFA) content ranging from 2–12% (w/w) of total fat. Dietary fat profiles were obtained by blending oils as previously described (30), and fatty acid composition of mixed diets was determined by gas chromatography (Table 1). After rats consumed diets for 4 wk, they were decapitated, and hearts and brains were removed.

Experiment 2. Rats (n = 6 treatment) were randomly assigned to 20% (w/w) fat diets with either 3 or 15% (w/w) of total diet as 18:2n-6. The fatty acid profiles were designed to produce similar n-6/n-3 ratios (Table 2). Following 0, 1, 2, 4, or 6 wk, rats were decapitated and hearts and brains removed.

Isolation of mitochondria. Brain mitochondria were isolated according to the method of Whittaker and Barker (31). Freshly isolated brains were homogenized in 0.32 M sucrose and centrifuged at 3,000 × g at 1°C for 15 min. The supernatant was decanted and recentrifuged to obtain the P2 pellet containing mitochondria, synaptosomes, and myelin. This pellet was applied to a discontinuous sucrose gradient and centrifuged at 100,000 × g for 60 min at 1°C. The mitochondrial pellet was resuspended in 1 mL isolation medium containing 0.21 M mannitol, 0.07 M sucrose, 0.1 M EDTA, 0.01 M Epps, pH 7.4, and heparin (330 U/mL solution).

TABLE 1
Fatty Acid Composition of Experiment 1 Diets[a]

Fatty acid	Diets					
	1	2	3	4	5	6
	(% of total diet)					
16:0	5.72	3.14	4.78	3.74	4.28	2.30
16:1	0.62	0.28	0.46	0.28	0.40	ND[b]
18:0	4.10	0.10	2.52	2.30	2.48	1.62
18:1	7.36	13.90	7.58	7.04	6.18	2.90
18:2n-6	2.02	2.36	4.44	5.96	6.26	11.70
18:3n-3	0.16	0.20	0.22	0.68	0.38	1.46
SFA	9.82	3.24	7.30	6.04	6.76	3.92
MUFA	7.98	14.18	8.04	7.32	6.58	2.90
PUFA	2.18	2.56	4.66	6.64	6.64	13.16
n-6/n-3	12.62	11.80	20.18	8.76	16.47	8.01

[a]Fat was extracted from premixed diets, and fatty acid profiles were determined by gas chromatography; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
[b]ND = Not detected.

TABLE 2
Fatty Acid Composition of Experiment 2 Diets[a]

Fatty acid	3% 18:2n-6	15% 18:2n-6
	(% of total diet)	
16:0	5.15	1.42
16:1	0.40	ND[b]
18:0	3.22	0.50
18:1	8.34	2.57
18:2n-6	2.82	15.04
18:3n-3	0.07	0.47
SFA	8.37	1.92
MUFA	8.74	2.57
PUFA	2.89	15.51
n-6/n-3	41.40	32.00

[a]Fat was extracted from premixed diets, and fatty acid profiles were determined by gas chromatography. Abbreviations as in Table 1.
[b]ND = Not detected.

Heart mitochondria were isolated by the method of Schnaitman and Greenwalt (32). Freshly removed hearts were washed to remove excess blood and were then individually homogenized in 10 vol of cold isolation medium (as above). The homogenate was centrifuged at 500 × g at 1°C, filtered to remove suspended lipid droplets, then recentrifuged at 12,000 × g at 1°C. The mitochondrial pellet was washed with 10 mL of isolation medium containing 0.5% (wt/vol) heparin, recentrifuged, and suspended in 1 mL of isolation medium.

Fatty acid analysis. Lipids were extracted from membrane preparations by a modification of the method of Folch *et al.* (33). The CL fraction was separated by thin-layer chromatography (34), and fatty acid methyl esters were prepared by methylation with boron trifluoride (35). CL fatty acid methyl esters were analyzed by gas–liquid chromatography using flame-ionization detection (Vista 402; Varian, Palo Alto, CA). Fatty acids were identified by comparing their retention times with those of authentic standards (Nu-Chek-Prep, Elysian, MN). Results are expressed as percentage of total fatty acids.

Statistical analyses. The sample size was selected to give power = 0.8. Data were analyzed using SAS 6.03 (SAS Institute, Inc., Cary, NC). For all analyses, the acceptable level of significance was 0.5%. Experiment 1 data were analyzed by two-way analysis of variance (ANOVA) to examine the main effects of diet, organ, and their interaction. Within organ comparisons of fatty acid, data were analyzed by one-way ANOVA followed by Student-Newman-Keul's test for multiple comparisons (36).

Data for experiment 2 were first analyzed by a three-factor ANOVA with diet, organ, and time as main effects. Because of many significant three-factor interactions, two-way ANOVA was used to test for the following interactions: diet by organ, diet by time, and organ by time. One-way ANOVA followed by Student-Newman-Keul's test for multiple comparisons was performed on data from each organ at each time period to examine the effect of diet on cardiac and neural membrane CL fatty acid composition.

RESULTS

Experiment 1. Both diet and organ had significant main effects on CL fatty acid profiles (Table 3). In addition, there were diet by organ interactions for 20:4n-6 and other less metabolically important fatty acids, 20:2n-6 and 20:3n-6. Heart CL had greater levels of 18:2n-6 and PUFA, and lower levels of 18:1, 20:4n-6, 22:6n-3, saturated (SFA) and monounsaturated (MUFA) fatty acids in comparison to neural mitochondria (Fig. 1).

To more closely examine the effect of dietary fat on CL fatty acid profile, data for each organ were subjected to an analysis of the simple effects of diet (37). In brain, diets with greater amounts of 18:2n-6 resulted in a higher percentage of CL 18:2n-6 and PUFA, and a lower percentage of 18:1, 22:6n-3, and MUFA than diets with lesser amounts of dietary 18:2n-6 (Fig. 2). Percentages of neural CL 18:0, 20:4n-6, and SFA were not affected by dietary fat.

Dietary fat had a similar effect on heart CL fatty acid composition. Rats that consumed diets with a greater amounts of 18:2n-6 tended to have a higher percentage of CL 18:2n-6 and a lower percentage of 18:1, 20:4n-6, 22:6n-3, and MUFA compared to rats that consumed diets with lesser amounts of 18:2n-6 (Fig. 2). Dietary fat did not affect heart CL fatty acid profiles of 18:0, SFA, or PUFA.

It is important to note that the lack of significant organ by diet interaction for 18:2n-6 suggests that the proportional changes (i.e., slopes of the response) were similar for both tissues despite the absolute differences in CL 18:2n-6. That is, CL 18:2n-6 increased by 1.5- to 2-fold in each tissue when comparing diets with the highest and lowest PUFA content, suggesting that the tissues responded similarly to the dietary manipulation. Nevertheless, since baseline levels of CL 18:2n-6 differ substantially (see Fig. 3), the absolute increase in CL 18:2n-6 in heart was 17% compared to an 8% increase in brain.

Experiment 2. When the effects of diet, time, and organ were examined using three-way ANOVA, third-order interactions for membrane CL 18:0, 18:2n-6, 20:4n-6, 22:4n-6, 22:6n-3, SFA, and PUFA were observed (data not shown). This indicates that the effect of diet on CL fatty acid composition is dependent upon the organ, i.e., heart or brain, and duration of treatment, i.e., 0 to 6 wk. Analysis of the data also revealed several two-way interactions (data not shown). There was a significant diet by time interaction in heart CL 20:4n-6 and a significant organ by time interaction for 18:2n-6 and 20:4n-6 in rats fed the high 18:2n-6 diet, and for 20:4n-6 in rats fed the low 18:2n-6 diet.

TABLE 3
Main Effects of Diet, Organ, and Their Interaction on Mitochondrial Membrane Cardiolipin Fatty Acid Composition

Fatty acid	Diet	Organ	Diet by organ
		P-value	
18:0	N.S.[a]	N.S.	N.S
18:1	0.0001	0.0001	N.S.
18:2n-6	0.0001	0.0001	N.S.
20:4n-6	0.001	0.0001	0.002
22:4n-6	0.0004	0.0001	N.S.
22:6n-3	0.0001	0.0001	N.S.
SFA	N.S.	0.03	N.S.
U.I.[b]	N.S.	0.0001	N.S.

[a]N.S. = Not significant. [b]U.I. = Unsaturation index. Other abbreviation as in Table 1.

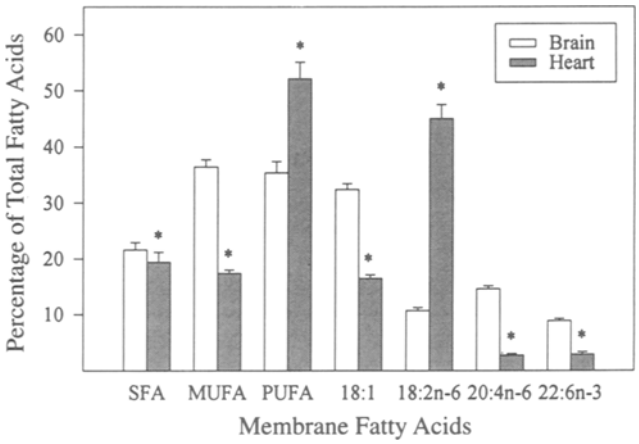


FIG. 1. Effect of organ on fatty acid composition of mitochondrial cardiolipin. Weanling rats were randomly assigned to 20% (w/w) fat diets with polyunsaturated fatty acid content ranging from 3 to 13% (w/w) of total diet. Diets were consumed for four weeks. Values are mean ± SEM; n = 10–11/treatment. An asterisk indicates a significant difference between organs ($P < 0.05$); SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

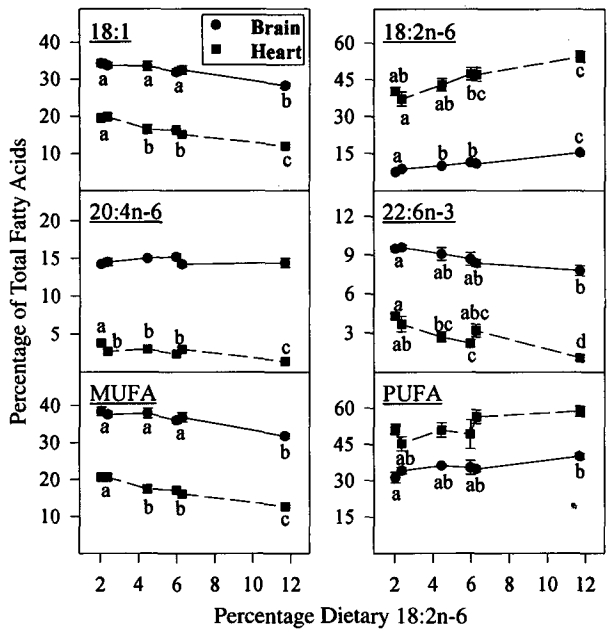


FIG. 2. Fatty acid composition of mitochondrial cardiolipin by organ and diet. Weanling rats consumed 20% (w/w) fat diets with 3 to 13% (w/w) polyunsaturated fatty acid content for four weeks. Values are mean ± SEM; n = 10–11/treatment. Values within the same organ and not sharing a common superscripted letter are significantly different ($P < 0.05$). Abbreviations as in Figure 1.

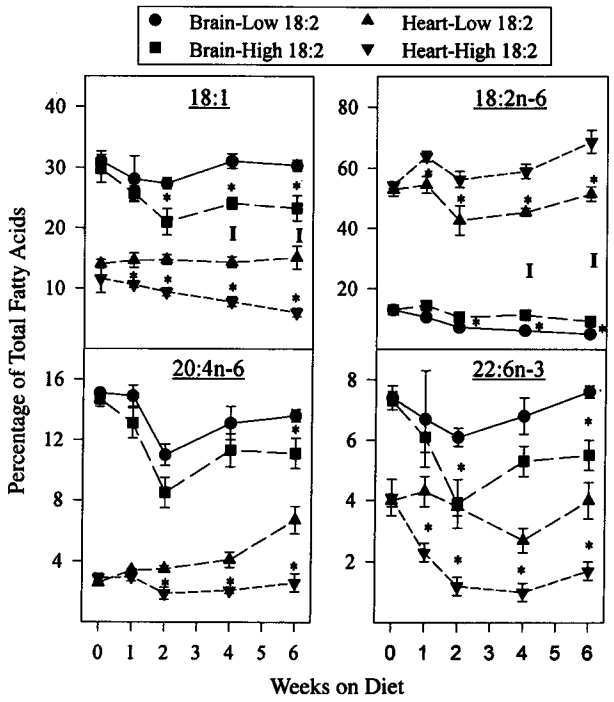


FIG. 3. Fatty acid composition of mitochondrial cardiolipin by organ and diet. Weanling rats consumed 20% (w/w) fat diets with either 3 or 15% of total diet as 18:2n-6 for one, two, four, or six weeks. Values are mean ± SEM; n = 6/treatment. An asterisk indicates a significant difference between diets within each organ and "I" indicates a significant diet by organ interaction ($P < 0.05$).

Examination of diet and organ effects within each time period revealed a significant interaction for CL 18:1 and 18:2n-6 after 4 and 6 wk of feeding the experimental diets (Fig. 3). In addition, there was a significant interaction term for PUFA at 6 wk (data not shown). An analysis of the simple effects of diet within each organ within each time revealed a significant diet effect (Fig. 3). Rats that consumed the high 18:2n-6 diet had greater levels of neural mitochondrial CL 18:2n-6 after 2, 4, or 6 wk than rats fed the low 18:2n-6 diet. In contrast, neural mitochondrial CL 18:1 was lower after 2, 4, or 6 wk in rats fed the high 18:2n-6 diet compared to rats fed the low 18:2n-6 diet. CL 22:6n-3 was lower in rats fed the high 18:2n-6 diets after 2 and 6 wk, whereas CL 20:4n-6 was lower in rats fed the high 18:2n-6 only after 6 wk.

Heart CL fatty acids showed a similar pattern of changes. Consistent with findings in experiment 1, the absolute differences between dietary treatments tended to be greater in heart mitochondrial CL fatty acids than in brain. CL 18:2n-6 was greater in hearts of rats fed the high 18:2n-6 diet for only 1 wk. This difference was evident at each subsequent time point. In contrast, heart mitochondrial CL 18:1 and 22:6n-3 were significantly lower in rats fed the high 18:2n-6 diet compared to those fed the low 18:2n-6 diet at each time point. In addition, CL 20:4n-6 was lower in rats fed the high 18:2n-6 diet at 2, 4, and 6 wk.

DISCUSSION

The results of this study demonstrated that both heart and brain mitochondrial membrane CL fatty acids are affected by dietary fat despite large biochemical and histologic differences in mitochondria from these two tissues. Importantly, the results suggest that mitochondria isolated from both tissues show similar sensitivity to dietary-fat induced alterations in CL fatty acid profile. That is, membrane CL levels of 18:2n-6 and PUFA increased, whereas the levels of 18:1, 22:6n-3, and MUFA decreased with increasing 18:2n-6 content (Fig. 2), and the magnitude (slope) of the response was similar in both tissues. When changes in CL fatty acid composition were examined periodically during a 6-wk interval, the response to dietary fat treatment tended to follow a similar pattern in both organs. Nevertheless, a significant effect of diet was noted somewhat earlier in cardiac tissue compared to neural tissue for 18:1, 18:2n-6, 20:4n-6, and 22:6n-3 (Fig. 3). These results suggest that changes in heart mitochondria display a faster time course in comparison to brain; however, brain mitochondrial CL fatty acid changes only lagged behind by 1 wk.

One objective of this study was to determine whether central and peripheral tissues would respond in a similar direction and similar degree to manipulations in dietary fatty acid composition. Previous work has demonstrated that mitochondria from a variety of peripheral tissues (liver, heart, kidney, and testes) responded differently to increasing dietary levels of 18:2n-6 (23). In general, CL 18:2n-6 levels increased in proportion to the dietary content of α-linoleate with CL from testes being notably resistant to the dietary manipulation. Our present data continue to show that brain mitochondrial CL is responsive to dietary α-linoleate content (19) and now suggest that the sensitivity of brain CL to dietary α-linoleate levels is comparable to that observed in CL isolated from cardiac mitochondria. That is, the proportional difference in CL 18:2n-6 between the diets with lowest and highest α-linoleate content was similar; nevertheless, the absolute difference in CL 18:2n-6 levels was greater in heart in comparison to brain.

There is a clear preference in heart mitochondria for the incorporation of 18:2n-6 into CL. α-Linoleate represents between 40 and 90% of total CL fatty acids depending upon the diet fed and is preferentially located in the 1(1″)- rather than the 2(2″)-position (23,25). In contrast, brain mitochondria has high levels of both 18:1 as well as the elongated EFA metabolites, namely 20:4n-6 and 22:6n-3. This overall profile of fatty acids in brain CL differs from that reported in testes (23) which also has low levels of 18:2n-6, but substitutes predominantly 16:0 rather than 18:1 in its place.

The degree to which this difference in fatty acid profile of CL from these various tissues represents differences in directed enzymatic incorporation of fatty acids or simply alterations in fatty acid supply to the mitochondria is uncertain. Like most other phospholipids, reacylation to obtain desirable fatty acid profiles is felt to occur post synthesis (38). However, unlike other phospholipids, synthesis of CL is be-

lieved to occur on the inner side of the mitochondria (39). Thus, fatty acid availability for reacylation will be governed by inner mitochondrial rather than cytosolic levels of fatty acids. Clearly the higher baseline levels of linoleate in mitochondria from most tissues is suggestive of greater preference for its incorporation into CL. Furthermore, studies demonstrating a preferential incorporation of *cis*-9,*cis*-12,*trans*-15 18:3 into CL in the absence of α-linoleic acid (26) also point to enzymatic specificity for 18:2n-6. The results of this study demonstrate that as dietary intake of α-linoleate increases parallel increases in α-linoleate incorporation into CL in brain occurs. This could suggest that brain mitochondria also has a preference for 18:2n-6, but that local supply is limiting its incorporation. This conclusion is supported by the fact that brain CL substitutes 18:2n-6 with 18:1n-9, 20:4n-6, and 22:6n-3 rather than 16:0 (as in testes) in an attempt to maintain overall chain length and unsaturation. Thus, it would appear that factors governing overall supply of fatty acids to neural tissues, and specifically to mitochondria within neural tissues, will have a specific effect on the fatty acid profile of brain CL.

Unlike many peripheral tissues, fatty acids that are components of phospholipids and triacylglycerides are not readily available to brain since they are not readily transported through the cerebral capillaries which form the anatomical basis of the blood brain barrier (40). Rather, plasma free fatty acids probably provide an important source of fatty acids to brain. Since free fatty acids are bound by high affinity sites on circulating albumin (40–42), only approximately 5% of plasma free fatty acids are unidirectionally taken up by the brain on a single pass through the cerebral microcirculation (41). Although recent studies have established that transport of SFA into the brain is protein-mediated (40,42), 18:2n-6 may cross the blood brain barrier by simple diffusion (40,42). Alternatively, 18:2n-6 may be taken up by target tissues *via* a receptor-mediated endocytosis. Such a mechanism has been suggested for retinal transport of 22:6n-3 (17). Although the precise mechanism for transport of EFA into brain is ill-defined at this time, these data collectively suggest that factors governing the plasma profile of free fatty acids, rather than those contained within lipoproteins, will have the greatest impact on brain fatty acid availability and, hence, mitochondrial CL fatty acid profile.

The time course of mitochondrial membrane changes observed in this study is consistent with our knowledge of its rate of turnover. The half-life of fatty acyl components has been reported to be about 3 d for fatty acids in CL obtained from peripheral tissues. Using this estimate, about 12 d of diet treatment would be required for greater than 90% turnover of phospholipid fatty acyl chains (21). Thus our data from cardiac CL is readily explained on the basis of this estimate. Nevertheless, it has generally been believed that the fatty acid turnover rate of brain membrane phospholipids is slower than that of peripheral tissues. This assumption has been based upon observations such as a lower rate of activity for CoA ligase, a microsomal enzyme that catalyzes the initial step for acylation of fatty acids. Additionally, experiments using an EFA deficiency model indicate that brain shows a slower rate of depletion of the EFA in comparison to peripheral tissues (1). Paradoxically, normal neural fatty acid profiles occur rapidly when EFA are restored to the diets of deficient rats. These observations could be consistent with a higher percentage of reutilization of fatty acids for reacylation reactions in brain in comparison to peripheral tissues rather than a decreased rate of phospholipid turnover. Such an argument would then be consistent with our present data suggesting, at least for CL, that the turnover rate of this phospholipid in brain and cardiac tissues does not differ substantively.

The degree to which these alterations in CL fatty acid profile are of physiologic relevance to the animal is ill-defined. However, CL is believed to be an integral part of some protein complexes where it may influence their conformation and hence activity (39). For example, cytochrome C oxidase, F_1-F_0-ATPase and the ADP/ATP carrier appear to be associated with CL (23,27). Changes in the affinity of CL to enzymes occur with changes in the unsaturation of the CL fatty acids. Furthermore, diets rich in sardine oil altered the fatty acid profile of cardiac CL and decreased the activity of cytochrome c oxidase and increased the activity of F_1-F_0-ATPase (27). These dietary effects on enzyme function may occur as a consequence of a perturbed fluid environment that affects protein aggregation, or due to changes in boundary lipids surrounding the membrane protein that affects conformation (2). Nevertheless, results from these studies suggest that the diet-induced alterations in CL profile may influence mitochondrial function, particularly as it relates to electron transport.

In summary, results from this study continue to demonstrate that brain CL fatty acid profile is sensitive to dietary fat consumption with notable changes in α-linoleate levels occurring. Despite large differences in baseline 18:2n-6 levels in neural and cardiac CL, the degree of sensitivity to dietary fat composition is comparable in terms of both the time course and degree of change observed. These results suggest that brain membranes continue to show sensitivity to dietary fat consumption even beyond periods of rapid brain growth and development.

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Blood Lipid Docosahexaenoic and Arachidonic Acid in Term Gestation Infants Fed Formulas with High Docosahexaenoic Acid, Low Eicosapentaenoic Acid Fish Oil

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ABSTRACT: The effect of fish oil high in docosahexaenoic acid (22:6n-3) and low in eicosapentaenoic acid (20:5n-3) in formula on blood lipids and growth of full-term infants was studied. Infants were fed formula with about 15% oleic acid (18:1), 32% linoleic acid (18:2n-6), 4.9% linolenic acid (18:3n-3) and 0, 0.10, or 0.22% 22:6n-3, or 35% 18:1, 20% 18:2n-6, 2.1% 18:3n-3 and 0, 0.11, or 0.24% 22:6n-3 from 3 d to 16 wk of age (n = 16,18,17,21,17,16, respectively). The formulae had <0.1% 20:5n-3 and no arachidonic acid (20:4n-6). Breast-fed infants (n = 26) were also studied. Plasma phospholipid and red blood cell (RBC) phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fatty acids were determined at 3 d and 4, 8, and 16 wk of age. These longitudinal analyses showed differences in blood lipid 22:6n-3 between breast-fed and formula-fed infants depending on the feeding duration. At 16 wk, infants fed formula with 0.10, 0.11% 22:6n-3, or 0.22% 22:6n-3 had similar 22:6n-3 levels in the plasma phospholipid and RBC PC and PE compared with breast-fed infants, and higher 22:6n-3 than infants fed formula without 22:6n-3. Formula with 0.24% 22:6n-3, however, resulted in higher plasma phospholipid 22:6n-3 than in breast-fed infants at 16, but not 4 or 8 wk of age. Plasma and RBC phospholipid 20:4n-6 was lower in formula-fed than breast-fed infants, but no differences in growth were found. Higher blood lipid C_{20} and C_{22} n-6 and n-3 fatty acids in infants fed formula with 20% 18:2n-6 and 2.4% 18:3n-3 compared with 32% 18:2n-6 and 4.9% 18:3n-3 show the increase in blood lipid 22:6n-3 in response to dietary 22:6n-3 depending on other fatty acids in the formula.
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Docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (20:4n-6) are long-chain polyunsaturated fatty acids which are present in cell membrane phospholipids and are important to many biochemical and physiological functions (1). Synthesis of 22:6n-3 and 20:4n-6 occurs *in vivo* by desaturation and elongation of the dietary essential fatty acids, linolenic acid (18:3n-3), and linoleic acid (18:2n-6), respectively (1–3). Human milk fatty acids usually contain 0.1 to 0.4% 22:6n-3, about 0.5% 20:4n-6 and variable amounts of 18:2n-6 and 18:3n-3 (4). Plasma and red blood cell (RBC) phospholipid levels of 22:6n-3 are lower in infants fed formula containing 18:3n-3 (but lacking 22:6n-3) than in most breast-fed infants (1,5–8). This has led to suggestions that 22:6n-3 should be added to formula, and results of several studies with term and preterm infants fed formula containing 22:6n-3 (9–15) have been published. In nonhuman primates and rodents, diets very low in 18:3n-3 (<0.1% energy) result in decreased brain and retina levels of 22:6n-3, altered learning abilities, and reduced visual function (1,16–18). In these species, a dietary source of 22:6n-3 does not seem to be necessary to achieve high brain and retina 22:6n-3 as long as the diet provides sufficient 18:3n-3. The importance of a dietary source of 22:6n-3 in healthy term infants fed with adequate amounts of 18:3n-3 is controversial (5,8,9,14,15,19,20). Despite this, it is reasonable to try to modify infant formulae to achieve blood lipid levels of 22:6n-3 in formula-fed infants which are similar to those of breast-fed infants.

Several studies have shown that plasma and RBC levels of 22:6n-3 in young infants can be increased by providing a dietary source of 22:6n-3 (10,11,15). In one study with premature infants, formula with 0.2% 22:6n-3 and 0.3% eicosapentaenoic acid (20:5n-3) from fish oil resulted in higher visual acuity scores at two and four months post-term, reduced blood lipid 20:4n-6, and lower growth and lower novelty preference scores and Bayley psychomotor, but not mental, developmental index scores than in premature infants fed formula without fish oil (11–13). It is not clear if these problems were due to the dietary 20:5n-3, or the relatively high total amount of n-3 fatty acids in the formula. Studies with piglets, however, have shown the amounts of 18:2n-6 and 18:3n-3 (and 18:1) in formula also influence blood and tissue levels of C_{20} and C_{22} n-6 and n-3 fatty acids (21). The present study, therefore, was undertaken to determine: (i) the amount of an oil high in 22:6n-3 and low in 20:5n-3 needed in formula to achieve a similar blood lipid 22:6n-3 to that of breast-fed infants, and (ii) if the amounts of 18:2n-6 and 18:3n-3 (and

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Abbreviations: ANOVA, analysis of variance; DHA, docosahexaenoic acid (22:6n-3); PC, phosphatidylcholine; PE, phosphatidylethanolamine; RBC, red blood cell.

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18:1) in formula alter the infant's blood lipid C_{20} and C_{22} n-6 and n-3 fatty acids. This was accomplished by comparison of the plasma and RBC phospholipid fatty acids and growth of term infants fed formula differing in 18:1, 18:2n-6, and 18:3n-3 and containing different amounts of a fish oil high in 22:6n-3 and low in 20:5n-3.

MATERIALS AND METHODS

Study design and subjects. This was a longitudinal study with term gestation infants fed one of six formulae using a 2 × 3 design, or breast-fed to 16 wk of age. Eligible infants were full-term (37–42 wk gestation), appropriate weight for gestational age, with 1 min Apgar scores greater than 7, and no known physical or metabolic disease, no history of intravenous lipid infusion or blood transfusion. Infants born to mothers with a history of diabetes, tuberculosis, or infection with known adverse effects on the fetus were excluded. Eligible infants were enrolled, monitored, and blood samples were obtained by the Philadelphia Association of Clinical Trials (PACT), Clinical Studies Inc., (Canton, OH), the Clinical Research Institute, (San Diego, CA), and the Children's Hospital of Philadelphia (Philadelphia, PA). Ethical approval for these studies was obtained from PACT Human Research Review Board, the Great Lakes Institutional Review Board, and Mercy Hospital and Medical Centre Institutional Review Committee, respectively. Written, informed consent was obtained from a parent for each infant prior to enrollment.

Infants who were not breast-fed were randomly assigned to one of the six formula groups within 3 d of birth using a randomization schedule developed centrally, which controlled for gender across the groups. Mothers were requested not to provide supplemental nutrients or foods, other than fluoride and water, to their infants during the study. Body weight, crown-heel length, and head circumference were measured at 3 d, and 4, 8, and 16 wk of age (5,6). Formula intake was obtained by parental recording for the 3 d immediately preceding each infant visit.

Formulas. The composition of the formulae was based on Similac® with Iron (Ross Products Division, Abbott Laboratories, Columbus, OH) with differences only in the fat blends (Table 1). Three formulae contained soy and coconut oil and were relatively low in 18:1, but high in 18:2n-6 and 18:3n-3 (high 18:2n-6 formulae). The other three formulae contained high-oleic safflower, soy and coconut oil, and were high in 18:1 but lower in 18:2n-6 and 18:3n-3 (low 18:2n-6 formulae). The formulae contained three different levels of a high 22:6n-3–low 20:5n-3 fish oil to provide 0.0, 0.10–0.11 and 0.22–0.24% 22:6n-3 in each of the fat blends. The formulae are abbreviated to indicate the fat blend and amount of 22:6n-3 as: high 18:2n-6—0.0%, 0.10 or 0.22% 22:6n-3, and low 18:2n-6—0.0%, 0.11 or 0.24% 22:6n-3. The ratio of 22:6n-3 to 20:5n-3 in the fish oil was approximately 4:1, resulting in less than 0.1% 20:5n-3 in all the formulas. Samples of breast milk were not obtained, but extensive analyses are available for North American women (4).

Blood sampling and analysis. Blood samples were collected by venipuncture from each infant at 3 d, and at 4, 8, and 16 wk of age. The plasma and RBC were separated by centrifugation, the RBC pellet washed, the samples coded, then immediately frozen and shipped on dry ice to Vancouver (6). Preliminary experiments were done to ensure that the methods of shipping resulted in delivery of frozen samples, and that there was no change in fatty acid composition of the samples during shipping or handling. Plasma and RBC lipids were extracted, and the plasma phospholipids and RBC phos-

TABLE 1
Oil Blend and Major Fatty Acid Composition of Formula[a]

	High 18:2n-6 formula			Low 18:2n-6 formula			Human
	0.0 DHA[b]	0.10 DHA	0.22 DHA	0.0 DHA	0.11 DHA	0.24 DHA	milk[c]
Oil							
Soy	60.0	59.5	59.1	23.0	23.0	23.0	
Coconut	40.0	39.6	39.4	37.0	36.2	35.5	
HO Saffl[d]	—	—	—	40.0	40.0	40.0	
Fish	—	0.8	1.5	—	0.8	1.5	
Fatty acids							
12:0	17.5	17.7	17.6	16.0	15.5	15.3	0.9–7.2
14:0	7.2	7.2	7.2	6.6	6.4	6.3	2.8–8.3
16:0	10.2	10.0	10.1	8.2	8.3	8.3	16.7–22.7
18:0	4.2	4.5	4.5	4.0	3.9	4.4	4.2–10.0
18:1	16.1	14.5	14.9	36.2	35.0	35.4	36.0–45.3
18:2n-6	34.2	32.2	31.9	20.5	20.0	20.4	7.0–22.4
18:3n3	4.8	5.0	4.9	2.1	2.2	2.1	0.7–2.6
20:5n-3	n.d.	<0.1	<0.1	n.d.	<0.1	<0.1	<0.1–0.1
22:6n-3	n.d.	0.10	0.22	n.d.	0.11	0.24	0.1–0.4

[a]Values are percentage total oil blend and percentage total fatty acids by weight. The following are not shown but represent 0.1 to 4% total fatty acids: 6:0, 8:0, 10:0, 16:1n-7, 20:0, 20:1, 22:0, and 24:0. The formulae had no 20 or 22 carbon chain n-6 fatty acids; n.d. indicates not detected, <0.1 indicates 0.0 to 0.05%.

[b]Docosahexaenoic acid (DHA) reflects percentage of 22:6n-3 in the formula.

[c]From 5, values are ranges for mature human milk, 20:4n-6 0.3–0.8%.

[d]HO Saffl, high oleic safflower oil.

phatidylcholine (PC) and phosphatidylethanolamine (PE) were separated by thin-layer chromatography and recovered (5,6). The fatty acids were converted to their respective methyl esters, separated and quantified using capillary column gas–liquid chromatography (22).

Statistical analysis. Continuous variables were analyzed using analysis of variance (ANOVA) to determine the effects of the diets on the plasma and RBC fatty acids. When significant differences were found, Tukey's multiple comparison procedure was used to determine which pairwise comparisons were statistically significant. Contrasts within the ANOVA were used to make comparisons for specific formula fat combinations: low 18:2n-6 compared with high 18:2n-6, irrespective of 22:6n-3, comparison of the formulae with 22:6n-3 compared with and without 22:6n-3, and of formulae with 0.10–0.11% compared with 0.22–0.24% 22:6n-3. Categorical (frequency) variables for formula tolerance, such as predominant stool color and predominant stool consistency, were analyzed by Chi-Square, and were repeated after sorting the data to correspond with the contrasts in the ANOVA procedures. The level of statistical significance was set at 5% for all analyses.

RESULTS

Of 206 infants enrolled, 131 successfully completed the study with 26, 16, 18, 17, 21, 17, and 16 infants in the breast-fed, high 18:2n-6 formula with 0.0, 0.10, or 0.22% 22:6n-3, and low 18:2n-6 formula with 0.0, 0.11, and 0.24% 22:6n-3 groups, respectively. There were no statistically significant differences in weight at birth or gender among the groups (Table 2). Fifty-six infants were withdrawn because of failure to complete the protocol (stopped breast feeding, $n = 12$; failure to attend an appointment for blood collection, $n = 29$; feeding >10% energy/d with formula for breast-fed babies or formula other than the study formula for formula-fed infants $n = 7$; illness and voluntary withdrawal, $n = 8$) and 19 infants were withdrawn by the infant's physician because of loose stools, diarrhea, rash, spitting-up, vomiting, crying, fussiness, gassiness, or other problems. There were no statistically significant differences in the incidence of the latter problems among the groups; 9 infants were fed formula with no 22:6n-3 and 10 were fed formula with 22:6n-3; 8 were fed a low 18:2n-6, and 11 were fed a high 18:2n-6 formula.

(i) Growth. There were no significant differences in weight, length, weight-to-length ratios, or head circumference, or standardized Z-scores for weight, length, or head circumference among the groups (Table 2) or in energy intake among the formula-fed groups (data not shown). However, it should be noted that in order to detect a difference of one standard deviation in the measures of growth with 80% power, this study would have required 30 infants per group.

(ii) Plasma and RBC phospholipid fatty acids. The fatty acid composition of the infants' plasma phospholipid, RBC PC, and RBC PE at 16 wk of age is shown in Tables 3–5, respectively. The changes in the blood lipid 22:6n-3 from 3 d to 16 wk of age are illustrated in Figures 1–3.

n-3 Fatty acids. At 16 wk of age, the infants fed formula had a significantly higher plasma phospholipid percentage 18:3n-3 and lower 22:5n-3 than infants fed human milk (Table 3). The RBC PC and PE percentage 18:3n-3 was also significantly higher in infants fed the high 18:2n-6 formulae than in the breast-fed infants (Tables 4 and 5). The RBC PE and PC percentage 18:3n-3 of infants fed the low 18:2n-6 formulae, on the other hand, was not different from that of infants fed human milk, except for a significantly higher percentage 18:3n-3 in the RBC PE of infants fed the formula with 0.0% 22:6n-3 (Table 4). Infants fed the formulae with no 22:6n-3 had significantly lower levels of 22:6n-3 in plasma phospholipids (Table 3) and RBC PE (Table 4) and PC (Table

TABLE 2
Characteristics of Infants at Start of the Study and at Sixteen Weeks of Age[a]

	Human milk	High 18:2n-6 formula			Low 18:2n-6 formula		
		0.0 DHA[b]	0.10 DHA	0.22 DHA	0.0 DHA	0.11 DHA	0.24 DHA
Birth	($n = 26$)	($n = 16$)	($n = 18$)	($n = 17$)	($n = 21$)	($n = 17$)	($n = 16$)
Gestational age (wk)	39.7 ± 0.2	39.9 ± 0.3	40.1 ± 0.1	39.9 ± 0.2	39.9 ± 0.1	39.9 ± 0.3	39.4 ± 0.2
Weight (kg)	3.45 ± 0.11	3.36 ± 0.13	3.31 ± 0.11	3.39 ± 0.09	3.56 ± 0.09	3.48 ± 0.16	3.30 ± 0.10
Male/female	12/14	7/9	9/10	8/9	10/11	8/8	7/9
Enrollment							
Weight-Z	0.12 ± 0.24	−0.01 ± 0.28	−0.23 ± 0.22	0.08 ± 0.20	0.49 ± 0.23	0.26 ± 0.32	−0.18 ± 0.21
Length-Z	0.09 ± 0.34	−0.06 ± 0.29	−0.32 ± 0.28	−0.22 ± 0.29	0.21 ± 0.23	−0.21 ± 0.20	−0.13 ± 0.29
Head circ-Z	−0.31 ± 0.20	−0.02 ± 0.36	−0.55 ± 0.20	−0.17 ± 0.15	−0.03 ± 0.26	−0.37 ± 0.29	−0.18 ± 0.32
Wt–length-Z	−0.45 ± 0.18	−0.45 ± 0.30	−0.31 ± 0.20	−0.37 ± 0.20	−0.07 ± 0.20	0.00 ± 0.33	−0.73 ± 0.26
16 Wk							
Wt-Z	0.13 ± 0.17	0.32 ± 0.31	0.30 ± 0.19	0.66 ± 0.20	0.59 ± 0.20	0.83 ± 0.19	0.32 ± 0.19
Length-Z	0.13 ± 0.18	0.29 ± 0.23	0.16 ± 0.31	0.24 ± 0.21	0.43 ± 0.24	0.44 ± 0.20	−0.00 ± 0.26
Head circ-Z	0.24 ± 0.13	0.21 ± 0.26	0.35 ± 0.18	0.40 ± 0.14	0.26 ± 0.22	0.35 ± 0.17	0.30 ± 0.28
Wt–length-Z	−0.06 ± 0.18	−0.01 ± 0.22	0.15 ± 0.23	0.49 ± 0.18	0.22 ± 0.23	0.46 ± 0.16	0.35 ± 0.19

[a]Values are means ± SE, with weight, length, and head circumference (circ) at enrollment and at 16 wk of age given as Z-scores. See Table 1 for abbreviation.
[b]Amount of DHA (% total fatty acids) in formula.

TABLE 3
Fatty Acid Composition of Plasma Phospholipids from Term Infants Fed Human Milk or Formula Varying in Oleic, Linoleic, Linolenic, and Docosahexaenoic Acid at Sixteen Weeks of Age[a]

Fatty acid	Human milk	High 18:2n-6 formula			Low 18:2n-6 formula		
		0.0 DHA[b]	0.10 DHA	0.22 DHA	0.0 DHA	0.11 DHA	0.24 DHA
	(*n* = 20)	(*n* = 12)	(*n* = 17)	(*n* = 11)	(*n* = 13)	(*n* = 10)	(*n* = 11)
18:3n-3[c]	0.1 ± 0.0	0.4 ± 0.0[f]	0.3 ± 0.0[f]	0.3 ± 0.0[f]	0.2 ± 0.0[f]	0.2 ± 0.0[f]	0.2 ± 0.0[f]
20:5n-3[d]	0.5 ± 0.1	0.4 ± 0.0	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.0	0.4 ± 0.1	0.6 ± 0.1
22:5n-3[c,d]	1.2 ± 0.1	0.8 ± 0.1[f]	0.7 ± 0.0[f]	0.8 ± 0.1[f]	0.6 ± 0.0[f]	0.6 ± 0.0[f]	0.6 ± 0.0[f]
22:6n-3[c,d,e]	4.3 ± 0.2	1.9 ± 0.2[f]	3.3 ± 0.1	4.6 ± 0.2	2.0 ± 0.1[f]	4.0 ± 0.2	5.2 ± 0.2[f]
18:2n-6[c]	21.4 ± 0.7	31.5 ± 1.0[f]	32.8 ± 0.3[f]	30.1 ± 0.9[f]	27.7 ± 0.5[f]	25.5 ± 0.5[f]	26.2 ± 0.7[f]
20:2n-6[c]	0.5 ± 0.0	0.7 ± 0.0[f]	0.7 ± 0.0[f]	0.6 ± 0.1[f]	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
20:3n-6[c]	2.6 ± 0.1	1.4 ± 0.1[f]	1.5 ± 0.1[f]	1.3 ± 0.1[f]	2.1 ± 0.1	2.1 ± 0.1	1.9 ± 0.1[f]
20:4n-6[f]	13.7 ± 0.5	8.0 ± 0.5[f]	7.4 ± 0.3[f]	8.3 ± 0.4[f]	8.6 ± 0.4[f]	8.5 ± 0.4[f]	8.5 ± 0.4[f]
22:4n-6	0.5 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.0	0.7 ± 0.0	0.7 ± 0.1	0.6 ± 0.1
22:5n-6	0.8 ± 0.1	0.3 ± 0.3[f]	0.3 ± 0.1[f]	0.3 ± 0.1[f]	0.6 ± 0.1	0.4 ± 0.1	0.5 ± 0.2
16:0	24.2 ± 0.3	24.1 ± 0.6	23.9 ± 0.4	25.1 ± 1.2	24.0 ± 0.6	24.3 ± 0.4	23.6 ± 0.5
18:0[c]	17.8 ± 0.2	19.4 ± 0.4[f]	19.0 ± 0.2[f]	18.1 ± 0.4	17.8 ± 0.3	17.5 ± 0.3	17.7 ± 0.3
18:1[c]	9.8 ± 0.3	6.9 ± 0.4[f]	6.4 ± 0.3[f]	6.3 ± 0.3[f]	12.1 ± 0.8[f]	12.4 ± 0.3[f]	11.3 ± 1.0
20:1[c]	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0

[a]Values given are means ± SE, 0.0 indicates value of <0.05 and >0.01. Values for 12:0, 14:0, 20:0, 22:0, 24:0, 14:1, 16:1, 22:1, 24:1, and 18:3n-6 are not shown. See Table 1 for abbreviation.
[b]Amount of DHA (% total fatty acids) in formula.
[c]Values for infants fed low 18:2n-6 formulae significantly different from values for infants fed high 18:2n-6 formulae, $P < 0.05$.
[d]Values for infants fed formulae with DHA different from infants fed same formulae without DHA, $P < 0.05$.
[e]Values for infants fed formula with 0.22 or 0.24% DHA different from values for infants fed the same formula with 0.10 or 0.11% DHA, $P < 0.05$.
[f]Values for group of infants fed formula significantly different from values for infants fed human milk, $P < 0.05$.

TABLE 4
Fatty Acid Composition of Red Blood Cell Phosphatidylethanolamine from Term Infants Fed Human Milk or Formula Varying in Oleic, Linoleic, Linolenic, and Docosahexaenoic Acid at Sixteen Weeks of Age[a]

Fatty acid	Human milk	High 18:2n-6 formula			Low 18:2n-6 formula		
		0.0 DHA[b]	0.10 DHA	0.22 DHA	0.0 DHA	0.11 DHA	0.24 DHA
	(*n* = 19)	(*n* = 9)	(*n* = 13)	(*n* = 9)	(*n* = 14)	(*n* = 7)	(*n* = 9)
18:3n-3[c,d]	0.1 ± 0.0	0.4 ± 0.0[f]	0.4 ± 0.0[f]	0.4 ± 0.0[f]	0.3 ± 0.1[f]	0.1 ± 0.0	0.1 ± 0.0
20:5n-3[d]	0.6 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.8 ± 0.1	0.4 ± 0.0	0.5 ± 0.0	0.7 ± 0.1
22:5n-3[c]	2.6 ± 0.2	2.2 ± 0.2	2.3 ± 0.3	2.8 ± 0.3	1.7 ± 0.2	1.9 ± 0.1	2.1 ± 0.2
22:6n-3[d,e]	5.9 ± 0.6	3.2 ± 0.3	4.9 ± 0.5	7.6 ± 0.8	2.8 ± 0.3	6.4 ± 0.5	8.2 ± 0.9
18:2n-6[c]	5.8 ± 0.3	12.5 ± 0.5[f]	11.6 ± 0.6[f]	11.1 ± 0.3[f]	7.7 ± 0.4[f]	7.5 ± 0.3[f]	7.3 ± 0.4
20:2n-6[c]	0.4 ± 0.0	0.9 ± 0.1[f]	0.9 ± 0.1[f]	0.7 ± 0.1[f]	0.7 ± 0.0[f]	0.8 ± 0.0[f]	0.7 ± 0.0[f]
20:3n-6	1.3 ± 0.1	1.6 ± 0.2	1.6 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.6 ± 0.2	1.4 ± 0.1
20:4n-6	23.9 ± 1.1	20.7 ± 1.0	18.7 ± 1.0[f]	19.3 ± 0.6[f]	18.4 ± 1.6[f]	20.3 ± 1.0	19.8 ± 1.2[f]
22:4n-6[c,d]	7.0 ± 0.5	6.6 ± 0.7	4.9 ± 0.4	5.3 ± 0.4	7.0 ± 0.5	6.8 ± 0.7	5.6 ± 0.6
22:5n-6[c,d]	1.4 ± 0.1	0.8 ± 0.1[f]	0.6 ± 0.1[f]	0.6 ± 0.1[f]	0.9 ± 0.1[f]	0.9 ± 0.1	0.9 ± 0.1
16:0[c]	18.7 ± 1.2	21.6 ± 1.9	20.0 ± 1.2	20.0 ± 1.2	20.0 ± 1.2	17.8 ± 1.1	18.8 ± 1.3
18:0[c]	8.6 ± 0.5	10.1 ± 0.9	10.3 ± 1.0[f]	9.3 ± 0.5	10.0 ± 0.8	8.0 ± 0.4	8.6 ± 0.6
18:1[c]	20.6 ± 0.8	16.7 ± 0.8[f]	18.4 ± 0.5	18.2 ± 0.7	25.0 ± 0.8[f]	25.5 ± 0.8[f]	22.9 ± 1.4
20:1[c]	0.7 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.4 ± 0.0	0.9 ± 0.2	0.7 ± 0.0	0.7 ± 0.1

[a]Values given are means ± SE, 0.0 indicates value of <0.05 and >0.01. Values for 12:0, 14:0, 20:0, 22:0, 24:0, 14:1, 16:1, 22:1, 24:1, and 18:3n-6 are not shown. See Table 1 for abbreviation.
[b]Amount of DHA (% total fatty acids) in formula.
[c]Values for infants fed low 18:2n-6 formulae significantly different from values for infants fed high 18:2n-6 formulae, $P < 0.05$.
[d]Values for infants fed formulae with DHA different from infants fed same formula without DHA, $P < 0.05$.
[e]Values for infants fed formula with 0.22 or 0.24% DHA different from values for infants fed the same formula with 0.10 or 0.11% DHA, $P < 0.05$.
[f]Values for group of infants fed formula significantly different from values for infants fed human milk, $P < 0.05$.

TABLE 5
Fatty Acid Composition of Red Blood Cell Phosphatidylcholine from Term Infants Fed Human Milk or Formula Varying in Oleic, Linoleic, and Docosahexaenoic Acid to Sixteen Weeks of Age[a]

Fatty acid	Human milk	High 18:2n-6 formula			Low 18:2n-6 formula		
		0.0 DHA[b]	0.10 DHA	0.22 DHA	0.0 DHA	0.11 DHA	0.24 DHA
	(*n* = 19)	(*n* = 8)	(*n* = 12)	(*n* = 10)	(*n* = 14)	(*n* = 7)	(*n* = 8)
18:3n-3[c]	0.1 ± 0.0	0.3 ± 0.0[f]	0.3 ± 0.0[f]	0.3 ± 0.0[f]	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:5n-3	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
22:5n-3[c]	0.4 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.0[f]	0.3 ± 0.1	0.2 ± 0.0
22:6n-3[d,e]	1.4 ± 0.1	0.7 ± 0.1[f]	1.0 ± 0.1	1.5 ± 0.1	0.6 ± 0.1[f]	1.4 ± 0.1	1.9 ± 0.3
18:2n-6[c]	17.7 ± 0.8	27.5 ± 0.7[f]	27.1 ± 1.3[f]	26.4 ± 0.8[f]	19.3 ± 1.3	21.5 ± 0.8	21.2 ± 1.3
20:2n-6[c,d]	0.4 ± 0.0	0.8 ± 0.0[f]	0.8 ± 0.0[f]	0.7 ± 0.0[f]	0.5 ± 0.0	0.6 ± 0.0[f]	0.6 ± 0.0
20:3n-6[c]	1.6 ± 0.1	1.2 ± 0.2	1.0 ± 0.1[f]	0.9 ± 0.0[f]	1.1 ± 0.1[f]	1.4 ± 0.1	1.1 ± 0.1
20:4n-6	6.7 ± 0.3	4.5 ± 0.2[f]	3.5 ± 0.2[f]	4.0 ± 0.3[f]	3.9 ± 09.4[f]	4.2 ± 0.3[f]	4.5 ± 0.4
22:4n-6	0.4 ± 0.0	0.7 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.2
22:5n-6	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0[f]	0.1 ± 0.0[f]	0.1 ± 0.0f	0.1 ± 0.0	0.1 ± 0.0
16:0	34.1 ± 0.7	33.0 ± 0.6	32.7 ± 1.1	34.3 ± 0.7	35.0 ± 1.0	33.4 ± 0.6	33.5 ± 1.3
18:0[c]	15.3 ± 0.3	16.2 ± 0.4	17.7 ± 0.6[f]	16.6 ± 0.3	15.6 ± 0.5	14.6 ± 0.3	14.9 ± 0.3
18:1[c]	18.6 ± 0.8	11.6 ± 0.4[f]	11.9 ± 0.5[f]	11.2 ± 0.5[f]	18.9 ± 0.7	18.1 ± 0.5	18.2 ± 0.7
20:1[c]	0.4 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.7 ± 0.1[f]	0.5 ± 0.1[f]	0.7 ± 0.2

[a]Values given are means ± SE, 0.0 indicates value of <0.05 and >0.01. Values for 12:0, 14:0, 20:0, 22:0, 24:0, 14:1, 16:1, 22:1, 24:1, and 18:3n-6 are not shown. See Table 1 for abbreviation.
[b]Amount of DHA (% total fatty acids) in formula.
[c]Values for infants fed low 18:2n-6 formulae significantly different from values for infants fed high 18:2n-6 formulae, $P < 0.05$.
[d]Values for infants fed formulae with DHA different from infants fed same formulae without DHA, $P < 0.05$.
[e]Values for infants fed formula with 0.22 or 0.24% DHA different from values for infants fed the same formula with 0.10 or 0.11% DHA, $P < 0.05$.
[f]Values for group of infants fed formula significantly different from values for infants fed human milk, $P < 0.05$.

5) than the breast-fed infants. In contrast, infants fed the formula with 0.1 or 0.11% 22:6n-3, or the high 18:2n-6 formula with 0.22% 22:6n-3 had plasma phospholipid, and RBC PC and RBC PE levels of 22:6n-3 which were not different from the breast-fed infants. Infants fed the formula with low 18:2n-6 and 0.24% 22:6n-3, however, had a significantly higher percentage of 22:6n-3 in plasma phospholipids than the breast-fed infants. The levels of 22:6n-3 in RBC PC and PE were also higher in infants fed the low 18:2n-6 formula with 0.24% 22:6n-3 than in the breast-fed infants, but the differences were not of statistical significance.

Infants fed the formulae with high 18:2n-6 had consistently higher levels of 18:3n-3 and 22:5n-3, and lower 22:6n-3 levels in plasma phospholipid, RBC PE, and RBC PC than infants fed the formulae with low 18:2n-6. Increasing the amount of 22:6n-3 in the formulae resulted in progressively higher 22:6n-3 levels in the plasma and RBC phospholipids (Tables 3–5, Figs. 1–3). Thus, levels of 22:6n-3 were significantly higher in infants fed formula with rather than without 22:6n-3, and higher in infants fed formula with 0.22 or 0.24% 22:6n-3 rather than 0.10 or 0.11% 22:6n-3. Levels of 20:5n-3, although significantly higher in the plasma phospholipid and RBC PE of infants fed the formulae with rather than without fish oil, were not significantly higher than in the breast-fed infants.

The difference in percentage 22:6n-3 in plasma phospholipid, RBC PE, and RBC PC between the breast-fed infants and infants fed formula with 22:6n-3 differed with the duration of feeding (Figs. 1–3). At 4 and 8 wk of age, infants fed the formula with 0.22 or 0.24% 22:6n-3 had plasma phospholipid and RBC PE and PC 22:6n-3 levels which were not different from that of the breast-fed infants. At 16 wk of age, however, the plasma phospholipid percentage 22:6n-3 was significantly higher in infants fed the formula with 0.24% 22:6n-3, but not different between infants fed the formula with 0.22, 0.11, and 0.10% 22:6n-3 and the breast-fed infants. Of importance, these longitudinal analyses show that the percentage 22:6n-3 in plasma and RBC phospholipid continues to change from 4 to 16 wk of age. The differences in blood lipid 22:6n-3 between breast-fed and formula-fed infants thus depend on the duration of feeding. These analyses also show that formula with about 20% 18:2n-6 and 2.1% 18:3n-3 results in consistently higher blood lipid levels of 22:6n-3 than formula with about 32% 18:2n-6 and 4.9% 18:3n-3.

n-6 Fatty acids. The levels of 18:2n-6 and 20:2n-6 in the plasma phospholipid, RBC PC, and RBC PE were significantly higher in infants fed formula with high (32%) 18:2n-6 than in infants fed human milk, or formula with low (20%) 18:2n-6. Infants fed the low 18:2n-6 formulae also had significantly higher plasma phospholipid and RBC PE 18:2n-6, and higher RBC PE and PC 20:2n-6 than infants fed human milk. In contrast to 18:2n-6 and 20:2n-6, the plasma phospholipid percentage 20:3n-6 was significantly lower in infants fed formula with high 18:2n-6 than in breast-fed infants, or in infants fed formula with low 18:2n-6.

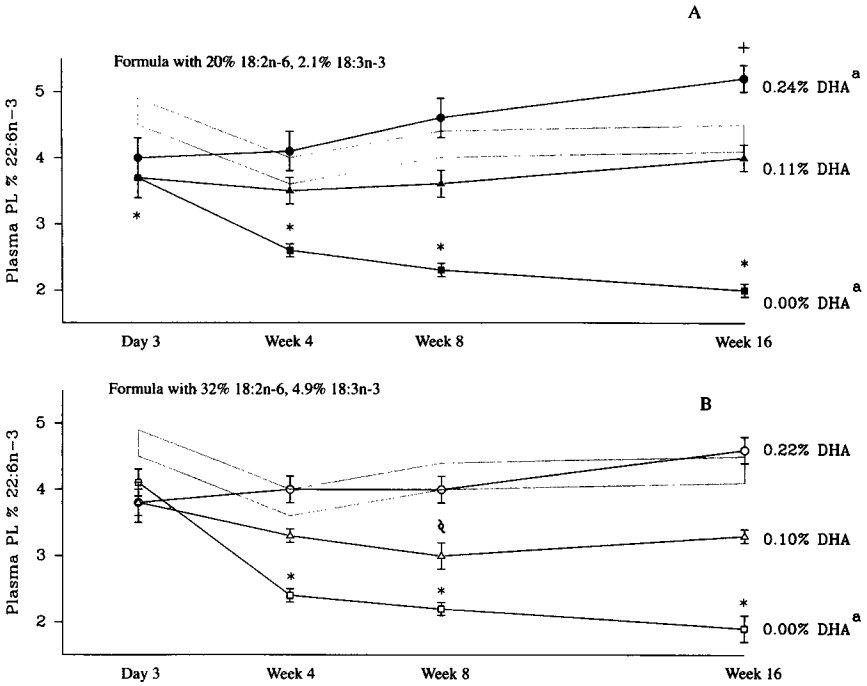


FIG. 1. Plasma phospholipid (PL) 22:6n-3 (% total fatty acids) of infants fed a formula with about (A) 20% 18:2n-6, 2.1% 18:3 or (B) 32% 18:2n-6, 4.9% 18:3n-3 and differing amounts of docosahexaenoic acid (DHA). Values given represent means ± SEM for each formula group. The mean ± SEM range for the group of breast-fed infants is indicated by the open box. The number of samples (infants) for the breast-fed infants and infants fed the 20% 18:2n-6 formula with 0.0, 0.11, or 0.24% DHA or 32% 18:2n-6 formula with 0.0, 0.10, or 0.22% DHA were: day 3, 16, 19, 15, 12, 11, 14, 15; wk 4, 21, 18, 11, 9, 12, 14, 12; wk 8, 21, 17, 11, 8, 11, 13, 12; wk 16, 20, 13, 10, 11, 12, 17, 11, respectively. The symbols *, §, and + indicate the value for infants fed 0.0%, or 0.10 or 0.11, or 0.22 or 0.24% DHA, respectively, is significantly different from the value for breast-fed infants, $P < 0.05$. The superscript a indicates value at 16 wk significantly different from value for the same infants at 4 wk, $P < 0.05$.

The infants fed formula had consistently lower levels of 20:4n-6 in plasma phospholipids (Table 3) and RBC PC (Table 5) than the breast-fed infants. The percentage 22:5n-6 was also significantly lower in the plasma phospholipids, RBC PE, and RBC PC of infants fed the formula with high 18:2n-6 than in the breast-fed infants, with the exception of the RBC PC in infants fed the high 18:2n-6 formula with 0.0% 22:6n-3. The percentage 20:4n-6 in the plasma phospholipid, but not RBC PE or PC, was significantly lower in infants fed formula with high rather than low 18:2n-6. Levels of 22:4n-6 and 22:5n-6, on the other hand, were significantly lower in the RBC PE of infants fed formula with high 18:2n-6 rather than low 18:2n-6 (Table 4).

The amount of 22:6n-3 in the formula had no statistically significant effect on the n-6 fatty acid composition of the infants' plasma phospholipids, RBC PC, or RBC PE, except for a significantly lower RBC PE percentage 22:4n-6 and 22:5n-6 in infants fed the formula with rather than without 22:6n-3 (Table 5).

Saturated fatty acids. Despite the lower 16:0 in the formulae than in human milk (Table 1), the percentage 16:0 and 18:0 in plasma phospholipids and RBC PC and PE were not significantly different between the breast-fed and formula-fed infants, except for a significantly higher percentage 18:0 in the plasma phospholipids of infants fed the high 18:2n-6 formula with 0.0 or 0.10% 22:6n-3, and in RBC PC of infants fed the high 18:2n-6 formula with 0.1% 22:6n-3 than in infants fed human milk (Tables 3–5). The percentage 18:0 in the plasma phospholipid, RBC PC, and RBC PE was significantly lower in infants fed the formulae with low 18:2n-6 than in infants fed the formulae with high 18:2n-6. This occurred despite similar amounts of 18:0 (3.9–4.5% fatty acids) in the formula (Table 1). The percentage 16:0 in the RBC PE, but not PC or plasma phospholipids, was also consistently lower in infants fed the formula low in 18:2n-6 than in infants fed the formula high in 18:2n-6. Part of this difference may be due to the difference in 16:0 in the formulae (about 8.3% compared with 10% 16:0 in the low and high 18:2n-6 formulae, respectively). The 22:6n-3 content of the formula had no significant effect on the saturated fatty acid composition of the infant's plasma phospholipids, RBC PC, or RBC PE.

Monounsaturated fatty acids. Human milk usually has about 36–45% 18:1, similar to that in the low 18:2n-6 formulae (about 36% 18:1) and about 2-fold higher than in the high

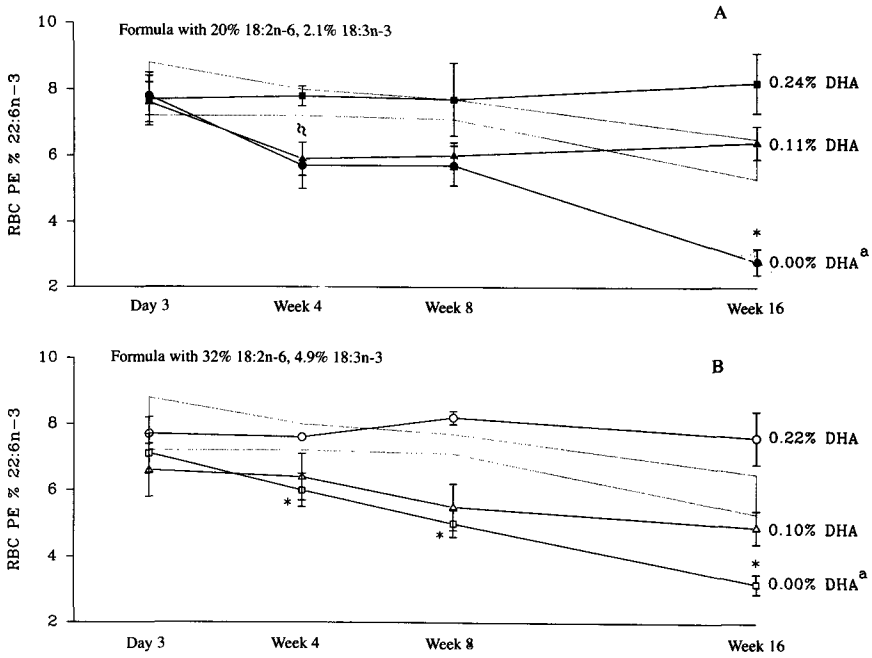


FIG. 2. Red blood cell phosphatidylethanolamine (RBC PE) 22:6n-3 (% total fatty acids) of infants fed a formula with about (A) 20% 18:2n-6, 2.1% 18:3 or (B) 32% 18:2n-6, 4.9% 18:3n-3 and differing amounts of DHA. Values given represent means ± SEM for each formula group where the SEM does not show the range is smaller than the symbol. The mean ± SEM range for the group of breast-fed infants is indicated by the open box. The number of samples (infants) for the breast-fed infants and infants fed the 20% 18:2n-6 formula with 0.0, 0.11, or 0.24% DHA or 32% 18:2n-6 formula with 0.0, 0.10, or 0.22% DHA were: day 3, 11, 9, 9, 8, 8, 10, 10; wk 4, 15, 8, 8, 6, 12, 10, 8; wk 8, 16, 9, 11, 6, 8, 10, 9; wk 16, 19, 14, 7, 9, 9, 13, 9, respectively. The symbols * and § indicate the value for infants fed 0.0 or 0.10% DHA, respectively, is significantly different from the value for breast-fed infants, $P < 0.05$. The superscript a indicates value at 16 wk significantly different from the value for the same infants at 4 wk, $P < 0.05$. See Figure 1 for other abbreviation.

18:2n-6 formulae (14.5–16.1% 18:1, Table 1). The plasma phospholipid and RBC PC of the infants fed formula with high 18:2n-6, and the RBC PE of infants fed the high 18:2n-6 formula with 0.0% 22:6n-3 had a significantly lower percentage 18:1 than in the breast-fed infants (Tables 3–5). The plasma phospholipids and RBC PE, but not RBC PC, of infants fed the formula with low 18:2n-6 with 0.0 or 0.11% 22:6n-3, however, had significantly higher levels of 18:1 than the breast-fed infants. The percentage 20:1 in the plasma phospholipids and RBC PC and PE was significantly higher in infants fed the formulae with low 18:2n-6 than in infants fed the formulae high in 18:2n-6. The 22:6n-3 content of the formula had no significant effect on the monounsaturated fatty acid composition of the infants' plasma or RBC phospholipids.

DISCUSSION

In the studies reported here, formula containing fish oil to provide similar amounts of 22:6n-3 and 20:5n-3 to that in mature human milk in North America (0.1–0.24% 22:6n-3, 22:6n3/20:5n-3 ratio of 4:1) resulted in higher blood lipid levels of 22:6n-3 than in infants fed formula without 22:6n-3. The plasma and RBC phospholipid percentage 20:4n-6 was lower in infants fed formula than in breast-fed infants, but was not further reduced by inclusion of 22:6n-3 in the formula. In contrast, studies with very premature infants have found lower levels of 20:4n-6 in plasma and RBC phospholipids of infants fed a formula with 0.2% 22:6n-3 and 0.3% 20:5n-3 than in infants fed formula with no 22:6n-3 and 20:5n-3 (11). Possibly, the absence of significant amounts of 20:5n-3, or the lower total amount of C_{20} and C_{22} n-3 fatty acids in the formulae explains the difference between the studies.

Evidence for retroconversion of 22:6n-3 to 20:5n-3 has been published (23). No consistent evidence was found in this study to suggest that increasing 22:6n-3 to up to 0.24% of formula fatty acids will increase the blood lipid 20:5n-3 or 22:5n-3 in healthy term gestation infants. Infants fed the formulae with 0.22 or 0.24% 22:6n-3 did not have higher plasma or RBC levels of 20:5n-3 or 22:5n-3 than infants who were breast-fed. This could be explained by the relatively low amount of 22:6n-3 in the formula (≤0.3% fatty acids), leading to incorporation of the dietary 22:6n-3 into tissue phospholipids rather than chain shortening.

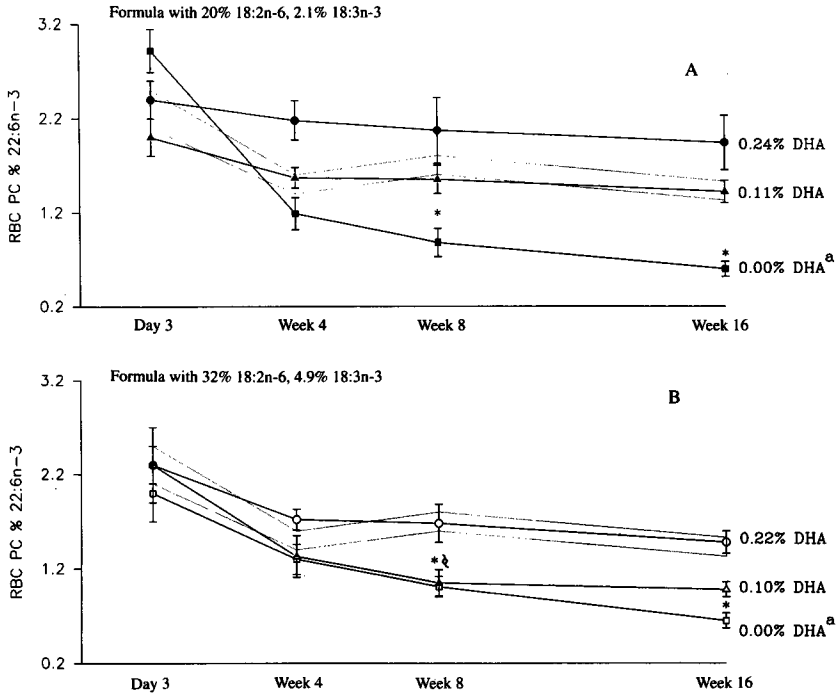


FIG. 3. Red blood cell phosphatidylcholine (RBC PC) 22:6n-3 (% total fatty acids) of infants fed a formula with about (A) 20% 18:2n-6, 2.1% 18:3 or (B) 32% 18:2n-6, 4.9% 18:3n-3 and differing amounts of DHA. Values given represent means ± SEM for each formula group. The mean ± SEM range for the group of breast-fed infants is indicated by the open box. The number of samples (infants) for the breast-fed infants and infants fed the 20% 18:2n-6 formula with 0.0, 0.11, or 0.24% DHA or 32% 18:2n-6 formula with 0.0, 0.10, or 0.22% DHA were: day 3, 11, 9, 8, 8, 9, 8, 10, wk 4, 17, 10, 7, 7, 11, 12, 8; wk 8, 17, 11, 9, 6, 8, 14, 10; wk 16, 19 14, 7, 8, 8, 12, 10. The symbol * or § indicates the value for infants fed 0.0% DHA or 0.11% DHA, respectively, is significantly different from the value for breast-fed infants, $P < 0.05$. The superscript a indicates value at 16 wk significantly different from value for the same infants at 4 wk, $P < 0.05$. See Figure 1 for other abbreviation.

Although no biochemical explanation is available, results of long-term studies with premature infants fed formula have suggested a relationship between blood lipid 20:4n-6 and growth (24). Despite differences in blood lipid 20:4n-6, no evidence of slower growth was found between the breast-fed and formula-fed term infants in the study reported here. Conclusions regarding growth, however, should not be drawn from this study because of the relatively small numbers of infants in each group. Other studies involving large numbers of infants, however, have found that formula-fed term infants grow at least as well as breast-fed infants (25). Whether the difference in blood lipid 20:4n-6 between term gestation breast-fed and formula-fed infants has any physiological significance is thus unknown.

In the studies reported here, infants fed the formula with about 32% 18:2n-6 and 4.9% 18:3n-3 had lower levels of plasma phospholipid 22:6n-3, 20:4n-6, and 20:3n-6 and higher 20:2n-6, than infants fed formula with about 20% 18:2n-6 and 2.1% 18:3n-3. These differences suggest that dietary intakes of 18:2n-6 and/or 18:3n-3 may reduce desaturation of 18:2n-6 to 20:3n-6 and 20:4n-6, with concomitant increased elongation of 18:2n-6 to 20:2n-6. Consistent with this, piglets fed formula high in 18:2n-6 had lower plasma, liver, and kidney, but not brain, phospholipid levels of 20:4n-6 and 22:6n-3 than piglets fed formula with lower 18:2n-6 (26). Evidence of reduced tissue 20:4n-6 and 22:6n-3 with increasing dietary 18:2n-6 also has been found in rodents (27). Other possible explanations include competition of 18:2n-6 with 20:4n-6 and 22:6n-3 for acylation to the 2-position of phospholipids. Although the explanation is uncertain, the findings suggest that the amount of 18:2n-6 and 18:3n-3 (or 18:1) in infant formula can determine in part the amount of 22:6n-3, and possibly 20:4n-6, needed to achieve blood lipid n-6 and n-3 fatty acid levels comparable to those in infants fed human milk. The longtitudinal analyses in this study also show that the age of the infant and/or the duration of feeding is important in determining differences in blood lipid n-6 and n-3 fatty acids between breast-fed and formula-fed infants.

In summary, this study has shown that the inclusion of fish oil to provide 0.10 to 0.22% 22:6n-3, with <0.1% 20:5n-3, in formula results in plasma and RBC phospholipid levels of 22:6n-3 in healthy term infants similar to those in infants fed human milk. The fish oil, which had a 22:6n-3 to 20:5n-3 ratio of about 4:1, had no significant adverse effects on

growth during the first four months after birth when added to give up to 0.24% 22:6n-3 in the formula fatty acids. The blood lipid 22:6n-3 response to dietary 22:6n-3 was consistently higher in infants fed formula with about 20% 18:2n-6 and 2.4% 18:3n-3 rather than 32% 18:2n-6 and 4.9% 18:3n-3. This suggests that the amount of 22:6n-3 required in formula to match the blood lipid levels of 22:6n-3 found in breast-fed infants will depend in part on the levels of other fatty acids in the formula.

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Gangliosides in Membranes from *Torpedo* Electric Organ

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ABSTRACT: The electric organ membrane has been the subject of many studies, due principally to its rich content of nicotinic acetylcholine receptor (AChR). Knowing its lipid composition is clearly important. Although its major membrane lipids have been characterized, its ganglioside composition has not been as well-described. In this study, gangliosides were characterized in membranes prepared from two species of electric organ, *Torpedo californica* and *T. nobiliana*. The ganglioside content of total electric organ membranes and AChR-enriched membranes was similar in both species, accounting for from 0.9 to 1.5% of membrane lipid by weight. However, the AChR-enriched membranes contained significantly less ganglioside relative to AChR than did the total membrane preparations. Five major gangliosides were purified from *T. californica* and identified as II^3NeuNAc-$GgOse_3$ (GM2); $II^3(NeuNAc)_2$-$GgOse_3$ (GD2), IV^3NeuNAc, II^3NeuNAc-$GgOse_4$ (GD1a), IV^3NeuNAc, $II^3(NeuNAc)_2$-$GgOse_4$ (GT1b), and IV^3-$(NeuNAc)_2$,$II^3(NeuNAc)_2$-$GgOse_4$ (GQ1b). Together these five gangliosides accounted for over 90% of the total ganglioside present in the two membrane preparations from both species. The most abundant ganglioside by far was GM2, which accounted for about one-half of the ganglioside content, followed by GD2. Determination of the N-fatty acid composition was performed on gangliosides purified from *T. nobiliana*. The lower-order gangliosides, GM2, GD2, and GD1a, contained substantial amounts of very long chain fatty acids (>20 carbons), including α-hydroxynervonic acid (15–21% of total). In contrast, unsubstituted, 14–18 carbon chains accounted for about 90% of the fatty acids on the two higher-order gangliosides, GT1b and GQ1b.

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Electric organ-derived membranes from various species of fish have been the subject of many studies on the nicotinic aceytylcholine receptor (AChR). Knowing their lipid composition is of major importance to understanding the structure and function of this protein *in situ*. For example, specific membrane lipids were found to be necessary in order to reconstitute full activity of the AChR in artificial membranes (1,2). Although the membrane content of phosphoglycerides and sphingolipids has been well-documented (for review see Ref. 3), the content of minor lipids, such as gangliosides, has not been as thoroughly investigated.

Some previous studies have described the ganglioside composition of electric organs and have revealed clear species differences. The electric organ from the bony fish, *Electrophorus electricus*, contained $II^3(NeuNAc)_2$-LC (GM3) as the only monosialoganglioside, as well as a substantial amount of Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc-(β1-1′)ceramide (GgOse4)-based higher-order (three or more sialic acid residues) gangliosides (4). In contrast, electric organ gangliosides from the bony fish *Gnathonemus petersi* did not contain significant amounts of monosialogangliosides, only higher-order gangliosides (5). The cartilaginous fish, *Torpedo marmorata*, appeared, on thin-layer chromatography (TLC), to contain the monosialogangliosides II^3NeuNAc,-II^3NeuNAc-$GgOse_4$ (GM1) and II^3NeuNAc-$GgOse_3$ (GM2) as major components with relatively smaller amounts of higher-order gangliosides (6–9). The ray, *Discopyge tschudii*, contained GM2 as the major ganglioside (55% of total), with lesser amounts of GM1 (7%), $II^3(NeuNAc)_2$-LC) (GD3) (10%), a ganglioside "running just below IV_3NeuNAc,-II^3NeuNAc-$GgOse_4$ (GD1a)" (12%), and higher-order gangliosides (13%) (5). A *T. californica* "membrane fraction" displayed a prominent band migrating "close to GM2" and a second major component with mobility between GD1a and $II^3(NeuNAc)_2$-$GgOse_4$ (GD1b) (10). Cholera toxin B binding after neuraminidase treatment showed that the majority of *T. californica* (10), *T. marmorata* (11), and *D. tschudii* (5) gangliosides did not contain the $GgOse_4$ oligosaccharide.

AChR-enriched, postsynaptic membranes isolated from *T. marmorata* contained GM1 as the major ganglioside (38% of total), with lesser amounts of GM2 (7%), GD3 (15%), a component running just below GD1a (20%), and higher-order gangliosides (less than 15%) (5). A separate study on *T. marmorata* AChR-enriched membranes also found that GM1 and GM2 predominated over higher-order gangliosides (11). *Discopyge tschudii* AChR-enriched membranes resembled those of *T. marmorata* except that GM2 was the major ganglioside (30%) in that species instead of GM1 (23%) (5). The ganglioside patterns of AChR-enriched membranes from both of

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Abbreviations: AChR, nicotinic acetylcholine receptor; FAME, fatty acid methyl esters: $GgOse_3$, GalNAc (β1-4)Gal(β1-4)Glc(β1-1′)ceramide; $GgOse_4$, Gal(β1-3)GalNAc(β1-4Gal(β1-4)Glc(β1-1′)ceramide; GD1a, IV^3NeuNAc,II^3NeuNAc-$GgOse_4$; GD1b, $II^3(NeuNAc)_2$-$GgOse_4$; GD2, $II^3(NeuNAc)_2$-$GgOse_3$; GD3, $II^3(NeuNAc)_2$-LC; GM1, II^3NeuNAc-$GgOse_4$; GM2, II^3NeuNAc-$GgOse_3$; GM3, II^3NeuNAc-LC; GQ1b, $IV^3(NeuNAc)_2$,-$II^3(NeuNAc)_2$-$GgOse_4$; GT1b, IV^3NeuNAc,$II^3(NeuNAc)_2$-$GgOse_4$; LC, Gal(β1-4)Glc(β1-1′)ceramide; TLC, thin-layer chromatography; TMS, trimethylsilyl.

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these species resembled those from the whole organ, except that enrichment of GM1 at the expense of GM2 was seen (5). The ganglioside content and composition of *T. marmorata* AChR-enriched membranes were very different from that of presynaptic membranes (11). Presynaptic membranes contained about fifteen times more lipid-bound sialic acid relative to protein than AChR-enriched membranes and were almost free of monosialogangliosides, but were enriched in higher-order gangliosides.

It should be noted that these previous studies have identified electric organ gangliosides based exclusively on the TLC mobility of native or neuraminidase-treated, cholera toxin B-labelled gangliosides. The only exception to this was the cholinergic-specific antigen, Chol-1β, which was purified from *T. marmorata* and found to be a novel trisialoganglioside that accounted for less than 0.1% of the total (9). This paper is the first structural analysis on purified major electric organ gangliosides and was undertaken to identify and quantify the major gangliosides in membrane preparations from the electric organs of the Pacific ray, *T. californica*, possibly the most frequently-used source of AChR-enriched membranes, and the Atlantic ray, *T. nobiliana*.

Furthermore, nothing is known about the fatty acid composition of electric organ gangliosides. The N-fatty acid composition of the major gangliosides purified from *T. nobiliana* electric organ is reported here for the first time.

MATERIALS AND METHODS

Preparation and characterization of membranes. Total and AChR-enriched membranes were prepared from frozen *T. californica* (Pacific Biomarine, Venice, CA) and *T. nobiliana* (Biofish Associates, Boston, MA) electric organ as previously described (12). Membrane preparations were assayed for protein content (13) and for [^{125}I]-α-bungarotoxin binding sites (DuPont-New England Nuclear, Boston, MA) (12). On average, the total and AChR-enriched membranes contained 0.4 and 0.9 nmoles of α-bungarotoxin binding sites per mg of membrane protein, respectively.

Ganglioside isolation and purification. Gangliosides were extracted from membrane preparations and partitioned into the aqueous phase following a previously described method (14). A sample of the total lipid extract was taken prior to partitioning for gravimetric determination of total lipids. The concentrated aqueous phase was applied to C_{18} reverse-phase cartridges (Millipore, Milford, MA) and washed with several volumes of distilled water followed by elution with methanol (15). The dried residue was solubilized in chloroform/methanol/water (60:30:4.5, by vol), filtered, and then applied to a DEAE-Sephadex A-25 ion exchange column (Pharmacia, Uppsala, Sweden) (16). The column was washed with methanol and then eluted with 0.5 M ammonium acetate in methanol. This sample was desalted on C_{18} cartridges, made 0.1 M in NaOH, heated at 45°C for 4 h to hydrolyze contaminating acidic phosphoglycerides (17), and then desalted again on C_{18} cartridges. TLC analysis showed that this sample contained no detectable free fatty acids or other contaminating lipids. Ganglioside recovery was not quantified in these studies but has been shown to be over 90% using this method (14). Individual gangliosides were purified on 500 μm silica gel TLC plates developed with chloroform/methanol/water (50:45:1, by vol) and eluted with chloroform/methanol/water (5:5:1, by vol), followed by washing on C_{18} cartridges.

Ganglioside characterization. Lipid-bound sialic acid was determined by the method of Svennerholm (18) and sphingosine by the method of Lauter and Trams (19). Analytical TLC was performed on 150 μm silica gel high-performance TLC plates (Alltech, Deerfield, IL) and developed with chloroform/methanol/aqueous 0.2% $CaCl_2$ at different volume ratios (see figure legends). Densitometric scanning at 580 nm of TLC plates stained for sialic acid was performed on a Beckman (Fullerton, CA) DU7 spectrophotometer.

For mild acid desialidation of gangliosides, a dried sample containing approximately 200 μg sialic acid was heated in 500 μL of 1 M formic acid in a stoppered Teflon-lined vial at 90°C for 1.5 h (20). The sample was dried under N_2, resuspended in upper phase, and washed on a C_{18} cartridge. Lipids were eluted with methanol and characterized by TLC.

For enzymatic desialidation, a dried ganglioside sample containing approximately 200 μg sialic acid was incubated at 38°C in 450 μL of 0.05 M sodium acetate at pH 5.0 containing 0.01 M $CaCl_2$ and 50 mU of *Clostridium perfringens* Type X neuraminidase (Sigma, St. Louis, MO) (21) either for 2 h (partial enzyme treatment) or for 18 h with an additional 50 mU of enzyme added at 12 h (complete enzyme treatment). Samples were handled as for mild acid desialidation. Parallel blanks containing enzyme, but no added ganglioside, were run to assure the enzyme preparations did not contain glycolipid contaminants.

Fatty acid and sugar composition were analyzed by gas chromatography/mass spectrometry (GC/MS) of their trimethylsilyl (TMS)–methyl esters and TMS–methylglycosides, respectively. The purified ganglioside was subjected to methanolysis in 0.1 mL of 0.9 M anhydrous methanolic HCl at 90°C for 16 h in a 1-mL sealed vial. After cooling, 300 μL of hexane was added and mixed well for 1 min. The hexane phase containing the fatty acid methyl ester (FAME) was removed to a clean 1-mL vial, and both phases were evaporated under N_2 in a 40°C water bath. The sugar methylglycosides sample was then dissolved in 100 μL of methanol to which 2 μL of pyridine and 10 μL of acetic anhydride had been added. This was allowed to stand at room temperature for 15 min to re-N-acetylate amino sugars and then dried under N_2. Both the FAME and sugar methylglycoside samples were then dissolved in 50 μL of pyridine/hexamethyldisilane-trimethylchlorosilane/bistrimethylsilyltrifluoroacetamide (2:1:1:2, by vol) and heated at 60°C for 30 min to form TMS–ethers of hydroxy–FAME and sugar methylglycosides. GC/MS was performed with a Finnigan 4500 MS system (Finnigan-MAT, San Jose, CA) equipped with a Hewlett-Packard (Avondale, PA) 5890 GC. From 1 to 3 μL of the TMS methylglycoside sam-

ples were injected *via* a dropping needle injector (Chrompac Inc., Raitan, NJ) maintained at 270°C. GC was on a 30 m by 0.25 mm i.d. DB-1 fused silica capillary column (J&W Scientific, Folsom, CA) with helium as the carrier gas. The column temperature was programmed from 190 to 250°C at 3°C/min and then to 270°C at 70°C/min, where it was held for 4 min. Hexoses eluted during the first ramp and sialic acids during the final hold. Ammonia chemical ionization was used with selected ion monitoring of the $[M + NH_4]^+$ ion from the neutral hexoses and the $[M + H]^+$ ions for N-acetylaminohexoses and sialic acids. FAME and TMS–hydroxyFAME were separated on a Hewlett-Packard 5890 gas chromatograph in the on-column injection mode with a flame-ionization detector on a 30 m by 0.25 mm i.d. DB-1701 column with 0.25 µm phase thickness. Helium was the carrier gas at 15 psi. Initial column temperature was held at 50°C and then programmed first to 200°C at 16°C/min and finally to 300°C at 4°C/min. All the FAME eluted during the final ramp. FAME from each ganglioside also were run on GC/MS and analyzed in the ammonia chemical ionization mode to confirm peaks seen on GC.

RESULTS

The lipid-bound sialic acid and ganglioside content of total and AChR-enriched membranes from both species studied are summarized in Table 1. The sialic acid content of total and AChR-enriched membrane preparations was about the same, although *T. californica* AChR-enriched membranes may contain slightly less lipid-bound sialic acid than total membranes relative to protein ($P < 0.1$). Because the AChR-enriched membranes contained over twice as much AChR, the amount of ganglioside relative to AChR in these membranes is lower than in the total membrane preparations.

TLC resolved the AChR-enriched membrane ganglioside fraction from *T. californica* into five major components (Fig. 1: A, C, D, E, and F). The same pattern was observed with AChR-enriched membranes from *T. nobiliana* and with total membranes from both species (not shown). Gangliosides purified from *T. californica* were used for structure determinations. The fastest ganglioside (A) moved just ahead of bovine brain II^3NeuNAc-$GgOse_3$ (GM2) on TLC, running as a wide band which often resolved into a doublet (Fig. 2). Sugar analysis revealed glucose, galactose, N-acetylgalactosamine, and N-acetylneuraminic acid at molar ratios of 1.0:0.8:0.8:0.9; N-acetylneuraminic acid was the only form of sialic acid detected. Mild acid hydrolysis generated one major product which displayed TLC mobility and staining behavior similar to bovine brain asialo-GM2 and, like the parent compound, often resolved into a doublet (Fig. 2); small amounts of lactocerebroside and glucocerebroside were also

TABLE 1
Ganglioside Content and Composition of Electric Organ Membrane Preparations

	Torpedo californica membranes		*T. nobiliana* membranes	
	Total	AChR-enriched	Total	AChR-enriched
Lipid-bound sialic acid[a]				
µg/mg Protein	3.1 ± 1.1	1.9 ± 0.5	1.9	2.1
µg/mg Lipid	5.1 ± 2.3	3.6 ± 0.7	3.2	2.4
Total gangliosides[b]				
nmole/mg Protein	5.2	3.6	3.6	4.8
nmole/mg Lipid	8.4	6.7	6.1	5.5
% Lipid (by wt)	1.5	1.1	1.0	0.9
nmole/nmole AChR	25.8	8.7	18.6	10.8
Major individual gangliosides (mole%)[c]				
GM2	41.6 ± 9.1	47.6 ± 8.6	53.1	64.9
GD1a	10.3 ± 2.7	10.8 ± 1.9	12.2	2.5
GD2	17.3 ± 2.8	13.6 ± 1.8	14.4	11.4
GT1b	17.3 ± 3.3	11.4 ± 4.8	9.6	8.9
GQ1b	9.6 ± 4.7	6.9 ± 2.0	6.4	3.7

[a]*Torpedo californica* values are expressed as the mean ± 1 SD from four samples (total membranes) or six samples [nicotinic acetylcholine receptor (AChR)-enriched membranes]. *Torpedo nobiliana* values are expressed as the average of two samples (total membranes) or that from a single sample (AChR-enriched membranes).

[b]Molar values were calculated from lipid-bound sialic acid values after estimation of the average sialic acid stoichiometries, which were derived from the quantification and structural determination of the major individual gangliosides. Ganglioside as percentage of lipid by weight was calculated after determining the average ganglioside molecular weights for each preparation.

[c]Gangliosides were identified as described in the text and then quantified by densitometric scanning at 580 nm of thin-layer chromatography plates stained for sialic acid followed by correction for sialic acid stoichiometry. *Torpedo californica* values are expressed as the mean ± 1 SD from four samples (total membranes) or three samples (AChR-enriched membranes). *Torpedo nobiliana* values are from single samples. GM2, II^3NeuNAc-$GgOse_3$; GD1a, IV^3NeuNAc, II^3NeuNAc-$GgOse_4$; GD2, $II^3(NeuNAc)_2$-$GgOse_3$; GT1b, IV^3NeuNAc, $II^3(NeuNAc)_2$-$GgOse_4$; GQ1b, $IV^3(NeuNAc)_2$-$II_3(NeuNAc)_2$-$GgOse_4$.

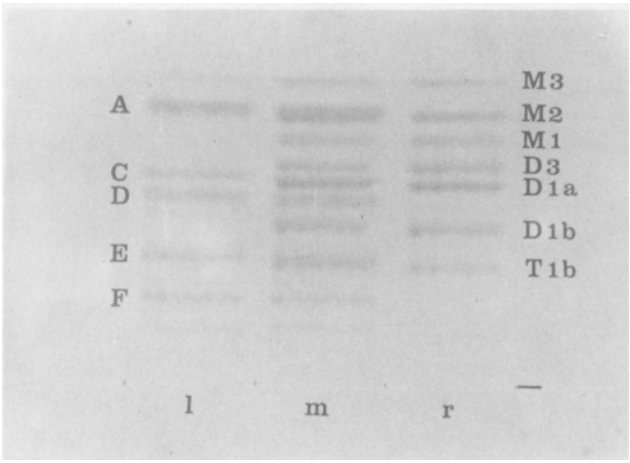


FIG. 1. Thin-layer chromatography of the total ganglioside fraction purified from *Torpedo californica* nicotinic acetycholine receptor-rich membranes (lane l). Lane r: bovine brain gangliosides; lane m: contents of lane l and lane r combined. Plate was developed with chloroform/methanol/aqueous 0.2% $CaCl_2$ (56:40:9, by vol) and visualized by spraying with 18 mM resorcinol and 0.25 mM $CuSO_4$ in concentrated HCl, followed by heating at 90°C for 15 min. All bands appeared violet.

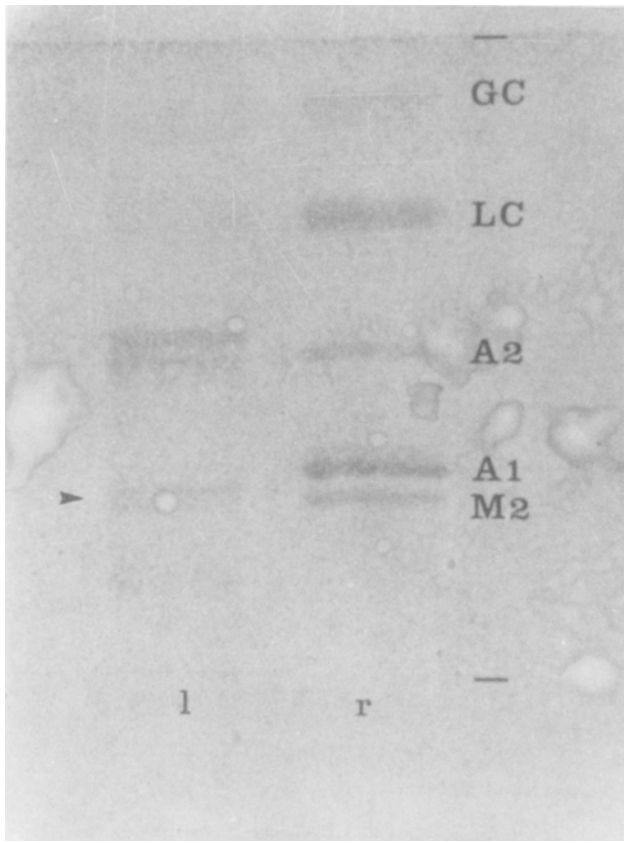


FIG. 2. Thin-layer chromatography of purified *Torpedo californica* ganglioside A following treatment with 1 M formic acid for 1.5 h at 90°C (lane l). Some unhydrolyzed A can still be seen (arrow). Lane r: untreated bovine brain lipids (A1: asialoGM1; A2: asialoGM2; LC: lactocerebroside; GC: glucocerebroside). Plate was developed with chloroform/methanol/aqueous 0.2% $CaCl_2$ (65:35:8, by vol) and visualized as described in Figure 1. Bands labelled with arrow and M2 appeared violet; all other bands appeared yellow or brown.

seen. Mild acid hydrolysis of bovine brain GM2 gave essentially the same results. These findings confirm that *Torpedo* ganglioside A is GM2.

Ganglioside C displayed TLC mobility just slightly faster than bovine brain $IV^3NeuNAc,II^3NeuNAc\text{-}GgOse_4$ (GD1a). The purified ganglioside had a sialic acid/sphingosine ratio of 2.17 and was degraded by neuraminidase to one major product, which comigrated with bovine brain GM1 (Fig. 3). These findings are consistent with ganglioside C being GD1a.

Ganglioside D always localized between bovine brain GD1a and GD1b on TLC. Like ganglioside A, it ran as a relatively wide band which often resolved into a doublet (Fig. 4). Purified ganglioside D had a sialic acid/sphingosine ratio of 2.06. It was degraded by neuraminidase to one major product which, like *Torpedo* GM2, migrated just ahead of bovine brain GM2 and also resolved into a doublet (Fig. 4). These findings indicate that ganglioside D is $II^3(NeuNAc)_2\text{-}GgOse_3$ (GD2).

Ganglioside E displayed TLC mobility similar to bovine brain $IV^3NeuNAc,II^3(NeuNAc)_2\text{-}GgOse_4$ (GT1b). Its sialic acid/sphingosine ratio was 3.10. Partial desialidation of E with neuraminidase generated a ganglioside comigrating with bovine brain GM1 as the major degradation product (Fig. 5); gangliosides comigrating with GD1b and GD1a were minor products. This same degradation pattern was seen with bovine brain GT1b. These findings indicate that ganglioside E is GT1b.

Ganglioside F consistently migrated just behind GT1b on TLC (Fig. 1) and corresponded closely with bovine brain $IV^3(NeuNAc)_2$, $II^3(NeuNAc)_2\text{-}GgOse_4$ (GQ1b) in mobility when run against total bovine brain gangliosides (not shown). Purified ganglioside F had a sialic acid/sphingosine ratio of 3.83, indicating that it is a tetrasialoganglioside. Formic acid treatment completely desialidated ganglioside F and gave two major products comigrating with asialoGM1 and lactocerebroside on TLC (not shown). These findings indicate that ganglioside F is most likely GQ1b.

Using these structural assignments, the relative molar amounts of the five major gangliosides were calculated from densitometry data (Table 1). On a molar basis, the most abundant ganglioside found in electric organ membranes from both species was GM2, accounting for roughly one-half of the ganglioside present. In fact, GM2 was the only monosialoganglioside found in substantial amounts. GD2 was the second most abundant ganglioside, so that $GgOse_3$-containing molecules made up about two-thirds of the ganglioside present. Together, the five major gangliosides accounted for more than 90% of the total ganglioside present in all membranes studied. The remaining sialic acid was distributed among several minor components, identified by TLC mobility as GM3, GM1, GD3, and an unidentified polysialoganglioside slower than GQ1b.

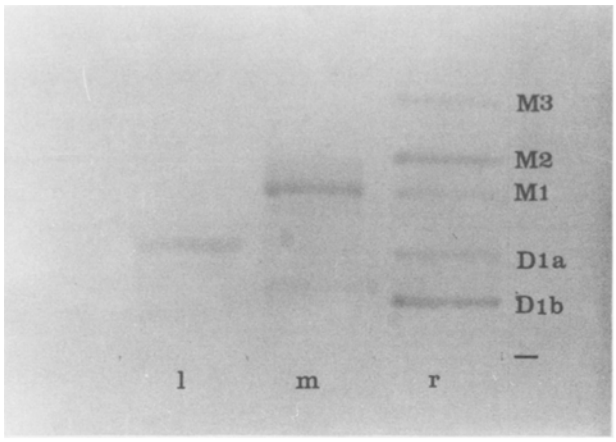


FIG. 3. Thin-layer chromatography of purified *Torpedo californica* ganglioside C following treatment with neuraminidase for 18 h at 38°C (lane m). Lane l: untreated ganglioside C; lane r: untreated bovine brain gangliosides. Plate was developed and visualized as described in Figure 1. All bands appeared violet.

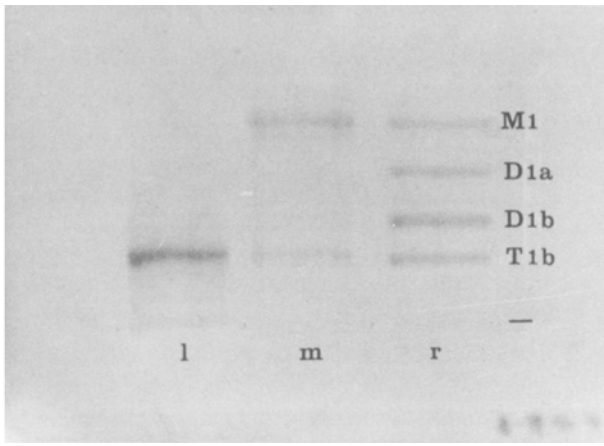


FIG. 5. Thin-layer chromatography of *Torpedo californica* ganglioside E following treatment with neuraminidase for 2 h at 38°C (lane m). Lane l: untreated ganglioside E; lane r: untreated bovine brain gangliosides. Plate was developed and visualized as described in Figure 4. All bands appeared violet.

Gangliosides purified from *T. nobiliana* electric organ were used to determine N-fatty acid content, which is summarized in Table 2. The majority of the fatty acids on all five major gangliosides were unsubstituted; however, α-hydroxynervonic acid [24:1(α-OH)], the only substituted fatty acid present in detectable amounts, accounted for 15–21% of the fatty acids on GM2, GD2, and GD1a. In contrast, the two higher-order gangliosides, GT1b and GQ1b, contained only 3% 24:1(α-OH). Unsubstituted fatty acids with chain lengths from 14 to 18 carbons predominated over those with 20 to 24 carbons in all five gangliosides; however, this was particularly evident among the higher-order gangliosides where 14 to 18 carbon fatty acids accounted for about 90% of their total fatty acid content. Saturated unsubstituted fatty acids predominated over monounsaturated chains on all five gangliosides; however, the unsubstituted fatty acids of GM2 and, to a lesser extent, GD2 and GD1a, did contain substantial amounts of monounsaturated chains with 22 or 24 carbons.

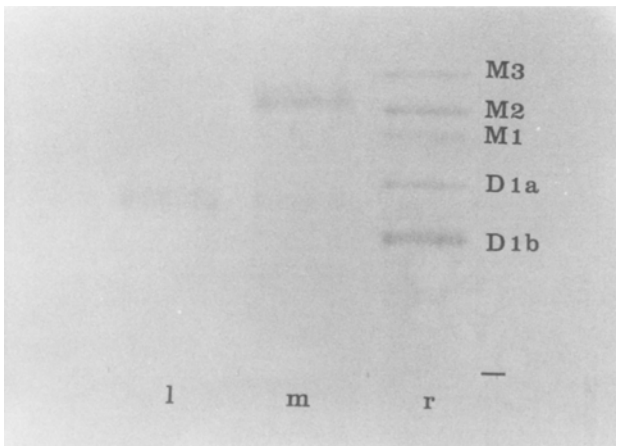


FIG. 4. Thin-layer chromatography of *Torpedo californica* ganglioside D following treatment with neuraminidase for 18 h at 38°C (lane m). Lane l: untreated ganglioside D; lane r: untreated bovine brain gangliosides. Plate was developed with chloroform/methanol/aqueous 0.2% $CaCl_2$ (50:45:10, by vol) and visualized as described in Figure 1. All bands appeared violet.

TABLE 2
Fatty Acid Composition of the Major Gangliosides from *Torpedo nobiliana* Electric Organ[a]

	Percentage of total fatty acids by weight in				
Fatty acid	GM2	GD1a	GD2	GT1b	GQ1b
14:0	9	8	7	8	9
15:0	1	1	1	3	2
16:0	17	23	24	33	33
16:1	3	6	7	15	10
18:0	11	17	11	14	18
18:1	5	6	9	18	14
Total C_{14-18}	46	61	59	91	86
20:0	3	3	4	1	1
22:0	4	6	ND	2	2
22:1	11	1	9	1	3
24:0	1	2	1	2	2
24:1	20	8	8	1	2
Total C_{20-24}[b]	39	20	22	7	10
24:1 (α-OH)	15	21	18	3	3
SFA/MFA[b]	1.2	2.9	1.5	1.8	2.3

[a]Fatty acids were determined by gas chromatography as described in the Materials and Methods section. Only fatty acids that accounted for 1% or more of the total are shown. Column totals may not equal 100% due to rounding; ND, not detected; other abbreviations as in Table 1.
[b]Values include unsubstituted fatty acids only; SFA/MFA, saturated/monounsaturated fatty acids.

DISCUSSION

The ganglioside content of the *T. californica* and *T. nobiliana* electric organs (0.9–1.5% of membrane lipid by weight) is similar to that reported for other extraneural tissues, for example, cardiac muscle (0.3–0.9%; Ref. 22) and liver (1.1–1.2%; Refs. 17,23). It is also similar to that estimated for *E. electricus* electric organ (0.7%; Ref. 4). The lipid-bound sialic acid content of the AChR-enriched membranes from *T. californica* (1.9 ± 0.5 μg/mg protein) and *T. nobiliana* (2.1 μg/mg protein) is similar to that reported for *T. marmorata* membranes (3.2 ± 0.2 μg/mg protein) (10). By comparison, these values are much lower than those of presynaptic electric organ membranes (10) and other nerve-derived tissue from this genus (6).

An important finding is the predominance of GM2 in *T. californica* and *T. nobiliana* electric organ membranes. A major band running "close to GM2" had also been noted in a *T. californica* membrane fraction (11). The predominance of GM2 is unusual, but not unprecedented, in an extraneural tissue. GM2 accounted for 55% of the gangliosides in the *D. tschudii* electric organ (5) and for 72% of the ganglioside present in eggs from the bony fish, *Mugil cephalus* (24). Interestingly, *T. marmorata* electric organ also contains GM2, but in relatively lower amounts (5,10).

This paper is the first to report finding substantial amounts (11–14%) of GD2 in the electric organ, although a prominent band between GD1a and GD1b can be seen in a membrane fraction from *T. californica* (11). It is quite possible that the major band from *D. tschudii*, which ran "just below GD1a," accounted for about 12% of the total and did not produce GM1 by neuraminidase treatment, may actually have been GD2 (5). Similarly, a prominent band between GD1a and GD1b in the gangliosides from *T. marmorata* did not appear to label with cholera toxin B following neuraminidase treatment and may also have been GD2 (10). The finding that the majority of *T. californica* (10), *T. marmorata* (11), and *D. tschudii* (5) electric organ gangliosides did not contain the $GgOse_4$ oligosaccharide supports these possibilities and suggests that an abundance of $GgOse_3$-based gangliosides may be a general feature of postsynaptic membranes from the electric organs of cartilaginous fish.

One of the most striking findings on AChR-enriched membranes from *T. californica* and *T. nobiliana* was the near absence of GM1. This contrasts with similar membranes from *D. tschudii* (5) and *T. marmorata* (5,10), where GM1 is a major component. Because *T. marmorata* presynaptic and synaptic vesicle membranes were found to be devoid of monosialogangliosides, it was even suggested that GM1 might be used to estimate postsynaptic contamination of presynaptic membrane preparations (10). Clearly, this criterion could not be used for *T. californica* or *T. nobiliana*, whose postsynaptic membranes are devoid of GM1. *Torpedo californica* and *T. nobiliana* also differed from *D. tschudii* and *T. marmorata* in their lack of GD3 (5). The present study reconfirms that there are important species differences in the ganglioside compositions of electric organ membrane preparations. These differences are not due to different methodological approaches, as the techniques used for preparing electric organ membranes and isolating the ganglioside fraction in these labs were very similar to the ones described in the present report (5,11).

The ganglioside patterns of *T. californica* and *T. nobiliana* indicate that both the "a" and "b" biosynthetic pathways are active in the electrocyte. The finding that the fatty acid patterns of the two higher-order gangliosides, GT1b and GQ1b, differed markedly from that of their putative precursor, GD2, suggests that extensive fatty acid remodeling may occur following their synthesis. Although no "c" pathway gangliosides were detected, they could still be present in minor amounts, as was seen with *D. tschudii* using monoclonal-antibody labelling (10).

The closely-spaced doublets seen on the chromatograms of GM2 and GD2 (e.g., see Figs. 2 and 4) may be due to their enrichment with 24:1(α-OH). Similar doublets seen on chromatograms of human liver GM3 were attributed to its α-hydroxyfatty acid content (25). The GM2 doublet cannot be due to different forms of sialic acid, as its desialidated derivative also resolved into a doublet (Fig. 2). The fatty acid compositions may also explain why *Torpedo* GM2 and GD1a appeared to move slightly faster than their bovine brain counterparts on TLC, and the higher-order gangliosides did not (see Fig. 1). The greater amounts of very long chain fatty acids on the lower-order gangliosides would tend to increase their mobility on TLC relative to the bovine standards, where 18 carbon fatty acids predominate. Relatively faster mobility was also observed with *T. marmorata* GM2 and GM1 (6,10), suggesting that the monosialogangliosides from that species may have a similar fatty acid pattern.

This study was not designed to determine if gangliosides interact with the AChR, but the results do allow for some speculation. The lower ratio of total ganglioside to AChR seen in the AChR-enriched membranes does not rule out a specific ganglioside-AChR interaction. However, if there were a strong interaction between a particular ganglioside and the AChR, one would expect membrane fragments enriched with AChR also to display enrichment with the ganglioside. This was not observed among the major gangliosides in *T. californica* and, although there is a suggestion of GM2 enrichment in *T. nobiliana*, the data is insufficient to draw this conclusion. This contrasts with *T. marmorata* and *D. tschudii*, whose AChR-enriched membranes were enriched with GM1 at the expense of GM2 (5). The present studies show that GM1 enrichment is not a general property of AChR-enriched membranes. This study's main contribution in this area is the definitive identification of candidate gangliosides on which to base future studies aimed at addressing the question of specific ganglioside–AChR interactions in the postsynaptic membrane.

ACKNOWLEDGMENTS

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Diversity of the Polar Lipids of the Food-Borne Pathogen *Listeria monocytogenes*

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ABSTRACT: *Listeria monocytogenes* is a Gram-positive bacterium that can adapt to high salinity and cold. Because the membrane lipids may play a role in its survival and adaptation, we have examined the polar lipids of *L. monocytogenes*. Extraction of total lipids from *L. monocytogenes* yielded 7 ± 1 mg/mL wet cells. Polar lipids represented 64% of total lipids and contained 9% lipid–phosphorus. Polar lipids were separated into 14 components by two-dimensional thin-layer chromatography. Eight components (88% of polar lipids) contained lipid–phosphorus; among these was one major component (34% of polar lipids). Two other phospholipids were ninhydrin-positive components and accounted for 15% of the polar lipids. Orcinol staining revealed two glyco- or sulfo-lipids accounting for 9% of polar lipids. Five components (4% of polar lipids) were amino components free of phosphorus. The major component contained 46% of its fatty acids as 15:0 *anteiso*, 24% as 17: 0 *anteiso*, and 11% as 15:0 *iso*. The fatty acid profile of the remaining polar lipids was variable, consisting primarily of 16:0, 18:0, 15:0 *anteiso*, and 17:0 *anteiso*. Their unsaturation level was ≤20%; however, the major phosphoaminolipid component was 46% unsaturated. The ratios of 15:0 *anteiso*/17:0 *anteiso* and 15:0 *anteiso*/15:0 *iso* were similar in all classes, averaging 1.5 and 4.5, respectively. Since the adaptation process to stressful environments involves activation of a membrane transport system for the protectant glycine betaine, the membrane lipids may play a role in enabling transport.
Lipids 31, 635–640 (1996).

Since the mid 1980s, *Listeria monocytogenes* has become recognized as a major cause of food-borne illness (1). This Gram-positive organism can cause septicemia, meningitis, and encephalitis, with a 20–40% mortality rate (2,3), in susceptible individuals such as pregnant women, the very old or very young, those with AIDS, those with neoplastic disease or renal collagen disease, and those undergoing immunosuppressant therapy. *Listeria monocytogenes* is found in low-acid cheeses, ice cream, milk, vegetables, raw and ready-to-eat meat, poultry (including eggs), and seafood (4). The organism is notably resistant to osmotic stress, and grows relatively well at temperatures as low as 1°C (e.g., 5). Therefore, food handling and storage practices, such as refrigeration, that suppress competitors can promote its growth.

The role of the membrane of *L. monocytogenes* in osmotic and chill adaptation is twofold. An immediate response to salt or chill stress by *L. monocytogenes* is the activation of a transport system for the protectant glycine betaine (2) from the medium. This response is particularly significant in the response to chill stress because low temperature is expected to lead to a phase transition of the lipids from liquid crystal to gel (lipid phase transition), which would not support normal membrane functions such as active transport. It appears that the presence of high levels of glycine betaine in the cytoplasm enables long-term adaptation processes to occur more readily. Chief among these adaptation processes is likely the restructuring of the membrane (e.g., 6–9). Thus the composition of the membrane of cells grown at mesophilic temperatures, which supports active transport when shifted to low temperature, is of paramount importance to the survival of the organism.

Previous studies by Von Graevenitz *et al.* (10) and Püttmann *et al.* (11) have been conducted on total fatty acid patterns for taxonomic classification of bacterial strains. These investigators reported changes in total fatty acids in response to growth at low temperature. In addition, Hether *et al.* (12) studied the immunological properties of cell wall preparations from a virulent strain of *L. monocytogenes*.

In 1971, Kosaric and Carroll (13) isolated the phospholipids of *L. monocytogenes* and obtained structural data with the aim of isolating and identifying the active substance that produced a "monocytosis" in rabbits similar to that seen in other animals infected with the bacterium (14,15). They reported that three of the major components are phosphatidylglycerol, diphosphatidylglycerol, and bisphosphatidylglycerolphosphate. Detailed analysis of the structure of these bacterial lipids has not been reported. This investigation was designed to analyze in detail the lipid classes, with the aim of targeting the interpretation of the distinct behavior of *L. monocytogenes*.

Our first step in understanding the dual role of the membrane in adaptation is to define in detail the composition of the membrane at mesophilic temperature. This paper reports the fractionation and separation of major polar lipid compo-

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Abbreviations: 1D-, 2D-HPTLC, one-dimensional, two-dimensional high-performance thin-layer chromatography; IR, infrared.

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nents, the fatty acid composition of the separated components, and suggestions for their structures.

MATERIALS AND METHODS

Culture of organism. Listeria monocytogenes strain DP-L1044 (D. Portnoy, University of Pennsylvania), a strain rendered avirulent by insertion of a transposon (16), in the gene encoding a virulence factor of the parent strain (Lm10403S), was grown in brain heart infusion (Difco Laboratories, Detroit, MI) broth at 30°C (*ca.* 24 h). Ten mL of this culture were used to inoculate one liter of brain heart infusion broth, which was then incubated at 30°C until late log phase. Cell density was determined by turbidity with a Klett-Summerson colorimeter with a green (No. 54) filter (209–212 Klett units). Cells were harvested by centrifugation at 5800 × *g* for 10 min and washed in 100 mM phosphate buffer, pH 7.0. Cells were washed again with the same buffer and harvested by centrifugation at 5,000 × *g* for one hour.

Extraction and isolation of polar from neutral lipids. Extraction of lipids was performed essentially according to the method of Folch *et al.* (17). After phase equilibration, the lower chloroform layer (total lipids) was evaporated to dryness, redissolved in chloroform/methanol (2:1, vol/vol), and stored at 0°C

Total lipids were fractionated by solid-phase extraction, using the method of Berger *et al.* (18) modified as follows. A lipid extract (~7 mg) was dissolved in 0.5 mL chloroform/hexane, 1:1, and applied to a pre-washed 100-mg silica column equipped with a stainless-steel frit and a 10-mL reservoir (Bond Elut; Varian Sample Preparation Products, Harbor City, CA). The columns were mounted on a Visiprep™ solid-phase extraction vacuum manifold (Supelco Inc., Bellefonte, PA). Neutral lipids were eluted three times with 6-mL aliquots of chloroform, and polar lipids were eluted with 7 mL methanol.

Chromatography of polar lipids. High-performance thin-layer chromatography (HPTLC) was carried out on precoated silica gel 60 plates (E. Merck, Darmstadt, Germany). For separation and detection of bacterial polar lipids, two-dimensional development (2D-HPTLC) was used with two solvent systems. System A 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). System B consisted of the following: dimension 1, chloroform/methanol/acetic acid/water (50:25:6:2, by vol); dimension 2, chloroform/methanol/28% ammonium hydroxide (65:35:5, by vol). Visualization of spots was effected by exposure to iodine vapor, followed by spraying with ninhydrin reagent and/or a phosphomolybdenum blue reagent (19). Lipid bands also were visualized using cupric sulfate spray and heating at 100°C for 2–5 min. The cupric sulfate reagent was 10% (wt/vol) cupric sulfate in 8% (by vol) phosphoric acid (20,21). An orcinol ferric chloride spray was used for detection of glyco- or sulfo-lipids (Sigma Chemical Co., St. Louis, MO). Tentative identification of the components was based on HPTLC mobilities relative to standards.

Lipid standards. The reference lipid standards bovine heart cardiolipin (sodium salt), L-α-phosphatidyl-DL-glycerol from egg yolk lecithin (sodium salt), L-α-phosphatidylcholine, β-arachidonyl-γ-stearoyl, L-α-phosphatidyl-L-serine from bovine brain, and dioleoyl L-α-phosphatidylethanolamine were purchased from Sigma Chemical Co.

Isolation and purification of individual polar lipids. Individual polar lipid components were isolated after separation by HPTLC with the two-dimensional solvent system (see A above). Spots were visualized by exposure to iodine vapors, and the individual spots were scraped and eluted from silica gel with 1 mL chloroform/methanol/water (1:2:0.8, by vol) three times. Two mL chloroform/water (1:1, vol/vol) were added to the solution, and after mixing, the chloroform extracts were evaporated and the residual lipids were used for further chromatographic analysis as well as for infrared (IR) and ^1H nuclear magnetic resonance spectroscopy. Individual spots were rechromatographed on one-dimensional (1D)- or 2D-HPTLC for confirmation of purity.

Analytical methods and quantitation of lipid components. Total phosphorus in the total combined lipids was determined by the method of Long and Staples (22). The polar lipids in bulk were quantitated by weight after elution from solid-phase extraction columns as described above. Individual polar lipid components were quantitated after development on 2D-HPTLC plates and staining with cupric sulfate by estimation of the spot intensities using a Bio Image Whole Band Analyzer (Millipore Corporation, Ann Arbor, MI).

Methanolysis of lipids' gas chromatography and gas chromatography/mass spectrometry analysis. Fatty acid methyl esters of individual polar lipid classes were prepared by scraping 2D-HPTLC spots directly into digestion tubes and converting the fatty acids to methyl esters with dry acid methanolysis using the method of Vance and Sweely (23) with the following modification: 400 µL of 3 M methanolic–HCl (10%, wt/vol; Supelco Inc.) reagent were added to each lipid sample, and methylation was allowed to proceed at 100°C for 5–6 h. Fatty acid methyl esters were extracted four times with 0.8 mL aliquots of petroleum ether. The samples were then dried and redissolved in hexane for gas chromatographic analysis. Fatty acid methyl esters were separated using a Varian 3400 gas chromatograph (Varian, Sunnyvale, CA) equipped with a septum-programmable injector, an on-column injector, and a flame-ionization detector. Methyl esters were separated on a DB™-23 30 m × 0.25 µm capillary column (J&W Scientific, Folsom, CA) with 50% cyanopropylphase, 0.25 µm film thickness. The carrier gas was He (head pressure 10 psi), and the make-up gas was N_2. The oven temperature was programmed from 90 to 130°C at the rate of 50°C/min and 130 to 210°C at 3°C/min. The injector temperature was programmed from 90 to 250°C at 70°C/min.

A standard mixture of bacterial fatty acid methyl esters (CP™ Mix catalog No.1114; Matreya, Inc., Pleasant Gap, PA) was chromatographed on two different capillary columns, a

DB™-23 (described above) and a DB™-5 20 m × 0.25 μm capillary (J&W Scientific) 5% phenyl column. Each peak of standard mixture was identified by mass spectrometry according to the method of Berger *et al.* (18). The fatty acid methyl esters of every unknown sample were chromatographed on the DB™-23 column and identified by comparison with the standard mixture. A confirmatory identification was performed by gas chromatography/mass spectrometry on the same column. Each peak with a retention time different from that of the standard was examined separately by gas chromatography/mass spectrometry on both the DB™-23 and DB™-5 columns, as was the standard.

Physical measurements. The isolated individual polar lipid components were further subjected to IR analysis. IR spectra were measured on thin liquid films between NaCl plates with a Perkin-Elmer (Norwalk, CT) 16PC FT-IR spectrometer.

RESULTS AND DISCUSSION

General observations. The total lipids of harvested *L. monocytogenes* cells extracted according to the procedure of Folch *et al.* (17), from four different cultures, amounted to 7 ± 1 mg/mL wet harvested cells, corresponding to approximately 7 ± 1 mg/100 mg dry cells. The lipid phosphorus content of the total lipids was approximately 5–6%. The polar lipids after separation from neutral lipids by solid-phase extraction amounted to 64% of total lipids, and the lipid–phosphorus content of the total polar lipids was approximately 9%.

By 2D-HPTLC separation in both solvent systems A and B, the most effective solvent was system A (dimension 1: acidic; dimension 2: neutral) (Fig. 1). In system B (dimension 1: acidic; dimension 2: alkaline), the components in the second dimension were concentrated for the most part near the solvent front.

The mobilities of the components of the total polar lipids were compared with those of cardiolipin, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine standards in a single HPTLC separation using chloroform/methanol/acetic acid/water (50:25:6:2, by vol) as the solvent system. The results indicated a component that co-chromatographed with phosphatidylglycerol and another that had an R_f lower than that of cardiolipin.

As determined by 2D-HPTLC ($n = 5$), the polar lipid fraction contained 14 components, which were designated as g_1, g_2, f_1, f_2, f_3, e_1, e_2, e_3, d, c_2, c_1, b, a, and those at the Origin (Fig. 1). The quantitative composition of this fraction is shown in Table 1. The major portion of the polar lipid components, components g_1, g_2, f_2, f_3, e_2, e_3, d, and b, accounted for 88% of the total and were phosphorus-positive. Of the phospholipids, components $g_1 + g_2$ were 55% of the total; $f_2 + f_3$, 15%; $e_2 + e_3$, 17%; and d and b, 5 and 6%, respectively. Among the phosphorus-positive components, components e_2 and e_3 were intensively ninhydrin-positive; thus phosphoaminolipids were 15% of the total. A small portion of the polar lipids of *L. monocytogenes* was aminolipid free of phosphorus (components e_1, c_2, c_1, a, and the Origin, which accounted for 3.7% of the total). Orcinol staining revealed two glyco/sulfo-lipids, components f_1 and a, which accounted for 8.7% of the total polar lipids.

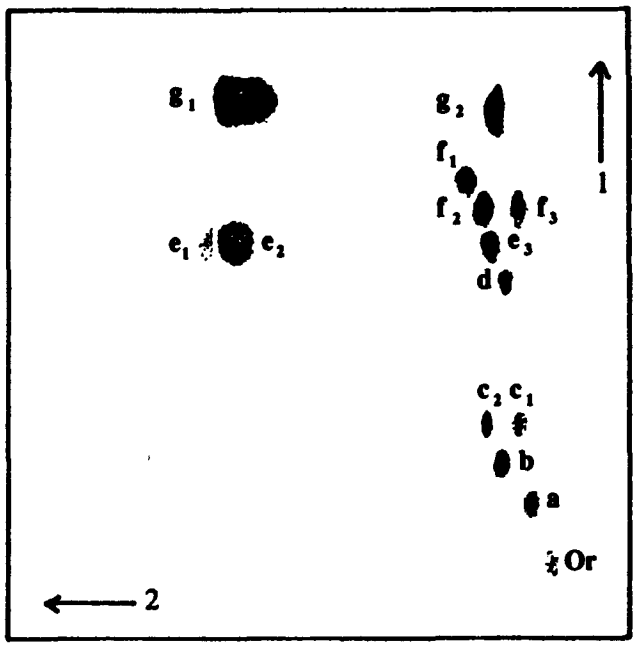


FIG. 1. Two-dimensional thin-layer chromatographic separation of polar lipids of *Listeria monocytogenes* on high-performance thin-layer chromatography silica gel 60. Plates were developed in direction 1 with chloroform/methanol/acetic acid/water (50:25:6:2, by vol) and in direction 2 with chloroform/methanol/water (65:20:3, by vol). The plate was stained by cupric sulfate/H_3PO_4 and charring. The letters a, b, c_1, c_2, d, e_1, e_2, e_3, f_1, f_2, f_3, g_1, g_2, and Origin (Or) denote the polar lipid components as described in the text.

The isolation and purification of each component was performed by separation of total polar lipids on a 2D-HPTLC with solvent system A, and after visualization by iodine vapor, the individual spots were scraped and eluted from silica gel as described above.

Fatty acid methyl esters identified after methanolysis are listed in Table 2. Generally, the fatty acids of the different polar lipid fractions exhibited notable differences in their relative complexities. Only component e_2 (11% of the total), which was an aminophospholipid, was characterized by high percentages of unsaturated fatty acids (47%), which was largely the 18:1n-9 *cis* species. In contrast, the major phospholipid component g_1 (34% of the total) had a negligible amount of unsaturated fatty acids. The remaining components, mainly the 18:1n-9 *cis* species, never exceeded 20% unsaturated fatty acids.

It is noteworthy that despite the differences in the relative complexity of the fatty acids of each component, the ratios of 15:0 *anteiso*/17:0 *anteiso* and 15:0 *anteiso*/15:0 *iso* were similar in all classes, averaging 1.5 and 4.5 , respectively. Nevertheless, the ratios of 17:0 *anteiso*/17:0 *iso* revealed a significant difference in their relative complexity. Also of interest is that the fatty acid profiles of the polar lipid components, with the exception of major phospholipid g_1, were characterized

TABLE 1
Quantitative Composition, HPTLC Mobilities, and Staining Behavior of Polar Lipid Components of *Listeria monocytogenes*[a]

Separated lipid component[b]	R_f values on HPTLC silica gel 60			Staining reagents			Percentage of total[c]
	C/M/AA/W (50:25:6:2)	C/M/W (65:20:3)	C/M/28% NH_3 (65:35:5)	Molyb. blue	Ninhydrin	Orcinol	
g_1	0.80	0.58	0.80	+			34
g_2	0.76	0.12	0.80	+			14
f_1	0.69	0.14	0.70			+	8
f_2	0.61	0.10	0.77	+			11
f_3	0.61	0.05	0.79	+			2
e_1	0.56	0.62	nd	i	+		trace
e_2	0.56	0.58	0.80	+	+		11
e_3	0.56	0.10	0.78	+	+		4
d	0.49	0.07	nd	+			5
c_2	0.27	0.10	0.71	i	+		1
c_1	0.27	0.03	nd		+		0.5
b	0.20	0.06	0.78	+			6
a	0.12	0.01	0.68		+	+	2
Origin	0.00	0.00	0.67		+		trace
Standard CL	0.85	0.16	0.82				
Standard PG	0.68	0.13	0.81				

[a]Abbreviations are: C, chloroform; M, methanol; AA, acetic acid; W, water; CL, cardiolipin; PG, phosphatidylglycerol; Molyb., molybdenum; i, indistinguishable; nd, not detected; trace (<0.4%).
[b]The polar lipids after separation by two-dimensional high-performance thin-layer chromatography (2D-HPTLC) on silica gel 60 plates were scraped and eluted as described in the Materials and Methods section. The letters denote each polar lipid component of *L. monocytogenes*.
[c]Components were quantitated as described in the Materials and Methods section.

primarily by the presence of four major saturated fatty acids, 16:0, 18:0, 15:0 *anteiso*, and 17:0 *anteiso*. The phospholipid component g_1 had a preponderance of the 15:0 *anteiso* species (see *The group of major phosphocomponents g_1 and g_2* section below).

Structural studies of the major polar lipids of L. monocytogenes. IR data showed a strong absorption band at 1730 cm^{-1}, indicating the presence of carboxylic esters in all major spots. Signficant information was obtained about each spot from chromatographic behavior.

The group of major phosphocomponents g_1 and g_2. Both components had the same (and the highest) R_f value on 1D-HPTLC in a chloroform/methanol/acetic acid/water (50:25:6:2, by vol) system but could be resolved by 2D-HPTLC into two phosphorus-positive spots, g_1 (higher R_f) and g_2 (lower R_f), in a chloroform/methanol/water system (65:20:3, by vol) (Fig. 1). This was considered to be due to differences in the nature of their constituent fatty acids, with the basic structure the same as that of beef heart cardiolipin as previously reported by Kosaric and Carroll (13). They indicated that 15:0 *br* (branched) plus 17:0 *br* species represent 78–100% of the fatty acids of polar lipids. Our results (Table 2) indicate that *L. monocytogenes* contains four major fatty acids (15:0 *anteiso* 49%, 17:0 *anteiso* 26%, 15:0 *iso* 10%, and 16:0 6%), which represent the 85% of fatty acid composition of polar lipids. It is in agreement with these results that species g_2 had HPTLC mobilities more similar to those of standard cardiolipin in the acidic, neutral, and alkaline solvent systems. Comparing these data with those of Kosaric and Carroll (13), it seems that g_1 plus g_2 might have overlapped when chromatographed in chloroform/methanol/water (65:25:4, by vol) and could have been identified as cardiolipin. Quantitatively the amount of components $g_1 + g_2$ is 55%, and the corresponding value of cardiolipin from Kosaric and Carroll (13) is 45%. Also, g_2 had 15.6% unsaturated fatty acids in contrast to species g_1, which contained a small amount of unsaturated fatty acids (see Table 2).

The g_1 species (the major polar lipid component, 34 .4%) contained 45.9% of its fatty acids as 15:0 *anteiso*, 23.7% as 17:0 *anteiso*, and 10.9% as 15:0 *iso*. The fatty acid composition of g_1 species was similar to that of component e_3 (see *The component group e_1, e_2, and e_3* section). The fatty acid profile of the g_2 species was appreciably different from that of the rest of the components, containing a variety of fatty acids but none in preponderance. Also, its unsaturated fatty acids included 5.6% 18:1n-9 *trans* fatty acid.

The component group f_1, f_2, and f_3. All of these components had the same R_f value on 1D-HPTLC in a chloro-

TABLE 2
Fatty Acid Composition (wt%) of Individual Polar Lipid Components of *Listeria monocytogenes*

Fatty acid	g_1[a]	g_2	f_1	f_2	f_3	e_1	e_2	e_3	c_2	c_1	b	a
2-OH 10:0	0.1											
12:0				1.1								
14:0	1.7	3.1	1.6	10.1	3.6	4.1	3.9	2.0	3.0	3.2	2.9	3.2
2-OH 14:0	0.0	1.9	0.1	0.6	1.1	0.9	0.3	0.1	0.3	0.3	0.3	0.4
3-OH 14:0		2.2										
15:0	0.2		0.5	1.1	3.0	2.6	2.4	0.4	1.5	1.9	2.3	2.2
i-15:0[b]	10.9	5.6	8.3	1.5	1.6	1.8	4.7	9.7	1.1	3.4	4.3	2.4
a-15:0[b]	45.9	20.9	29.8	5.2	10.3	8.4	18.0	41.2	5.7	18.6	20.0	13.0
16:0	8.2	21.2	11.7	31.9	26.2	26.1	5.8	10.0	29.1	22.2	19.7	22.3
i-16:0	1.9	2.0	2.8	0.5	1.3	2.3	1.1	2.1	0.1	2.9	1.6	4.2
2-OH 16:0	0.1	2.0	1.2	0.7	3.8	0.9	0.2	0.1	4.0	2.4	3.1	3.1
16:1n-7 *cis*		0.5		0.4								
17:0	0.5		1.4	1.3	11.0	7.3	0.9	1.2	7.3	5.2	7.0	7.8
i-17:0	2.9	2.9	3.1	0.9	0.5	2.6	1.9	3.1	1.3	2.1	1.8	2.0
a-17:0	23.7	11.8	32.5	2.9	6.9	6.3	8.4	21.8	6.2	12.1	18.2	9.8
18:0	2.6	8.9	3.7	19.5	17.3	16.7	3.0	4.8	17.1	14.0	10.7	13.3
18:1n-9 *cis*	0.7	9.5	1.8	3.0	7.2	12.5	46.4	1.8	11.6	6.3	4.4	9.7
18:1n-9 *trans*		5.7		14.3			0.1	0.2				
18:1n-7 *cis*				3.7								
18:2n-6 *cis*	0.7		1.1	0.6	5.0	6.7	0.2	1.3	8.5	4.3	3.0	5.0
19:0		1.9	0.2		0.6	0.2		0.1	0.5	0.3	0.3	0.4
19:0 cyclo			0.2	0.8	0.6	0.7	0.7	0.1	2.0	0.9		1.2
a-15:0/*a*-17:0	1.9	1.8	0.9	1.8	1.5	1.3	2.1	1.9	0.9	1.5	1.1	1.3
a-15:0/*i*-15:0	4.2	3.8	3.6	3.6	6.3	4.6	3.8	4.3	5.4	5.4	4.7	5.5
a-17:0/*i*-17:0	8.2	4.0	10.4	3.2	12.9	2.4	4.4	7.0	4.9	5.7	10.2	5.0
Total unsaturated	1.4	15.6	2.9	21.7	12.2	19.2	46.8	3.3	20.0	10.5	7.4	14.7

[a]The letters a, b, c_1, c_2, e_1, e_2, e_3, f_1, f_2, f_3, g_1, and g_2 denote the polar lipid components of *L. monocytogenes* as described in the text.
[b]*i*: *iso*, *a*: *anteiso*, *iso* and *anteiso* isomers of branched-saturated fatty acids.

form/methanol/acetic acid/water (50:25:6:2, by vol) system and could be resolved by 2D-HPTLC into three spots in a chloroform/methanol/water (65:20:3, by vol) system. One species, f_1, was free of phosphorus and was an orcinol-positive component (see below for *the components f_1 and a*). The other two components were phosphorus-positive molecular species, f_2 (higher R_f) and f_3 (lower R_f) in a neutral system (see Fig. 1). This separation in the second dimension was possibly due to differences in the nature of their constituent fatty acids. The components f_2 and f_3 were therefore tentatively identified as phosphatidylglycerols. This supposition was in agreement with the HPTLC mobilities of standard phosphatidylglycerol in the neutral, acidic, and alkaline solvent systems (Table 1). Phosphatidylglycerol is reported to be among the lipids of *L. monocytogenes* (13). In the present separation, we have used the solvent system chloroform/methanol/water (65:20:3, by vol) in the second dimension, which is of slightly different composition than that used by Kosaric and Carroll (13), chloroform/methanol/water (65:25:4, by vol). Components f_2 and f_3 are not well resolved when chromatographed in chloroform/methanol/water (65:25:4, by vol) may have been identified by Kosaric and Carroll (11) as phosphatidylglycerol.

The f_2 component contained palmitic acid (16:0) and stearic acid (18:0) as the predominant fatty acids, at 31.8 and 19.5%, respectively. This species had a higher proportion of unsaturated fatty acids (21.6%), which included 14.3% of 18:1n-9 *trans* fatty acids and a high percentage (10%) of the 14:0 species (Table 2).

The component group e_1, e_2, and e_3. The compounds e_2 and e_3 had the same R_f value on HPTLC in a chloroform/methanol/acetic acid/water (50:25:6:2, by vol) system but could be resolved by 2D-HPTLC into its three components. Species e_2 and e_3 were intensely phosphorus and amine-positive and were considered to be two molecular species differing only in the nature of their constituent fatty acids. Their HPTLC mobilities in the alkaline system were similar (see Table 1). Species e_2 was a major component of the total polar lipids (11%) and its predominant fatty acid moiety was 18:1n-9 *cis* (46.4%).

In the alkaline system, e_2 and e_3 had the same HPTLC mobility as the component g_2. Component e_1 was in trace quantity and only slightly separated from e_2; it was not possible to distinguish whether it was phosphorus-positive, but it was a ninhydrin-positive component. Of note, e_2 and e_3 were the only amino phospholipids of *L. monocytogenes* polar lipids, and they amounted to 15% of the total. The fatty acid composition of e_3 was indistinguishable from that of g_1 (see *The group of phosphocomponents g_1 and g_2*).

The components f_1 and a. These components are suspected

to be glyco- or sulfo-compounds because they were the only components that stained with orcinol reagent. Of note, component a was also an aminolipid (see also *The component group f_1, f_2, and f_3*).

Component f_1 had a preponderance of 17:0 *anteiso* and 15:0 *anteiso* fatty acid species (32.5 and 29.8%, respectively) and was characterized by a very low content of unsaturated fatty acids (2.8%). In contrast, the fatty acid profile of component a contained a variety of fatty acids. The following were the major species: 16:0, 18:0, 15:0 *anteiso*, 17:0 *anteiso*, 18:1n-9 *cis*, accounting for 22.3, 13.3, 13, 9.8, and 9.7%, respectively. Component a also contained substantial percentage (15%) of unsaturated fatty acids (Table 2).

The analysis of *L. monocytogenes* lipids revealed a complex lipid pattern; further studies are necessary to identify each component. Furthermore, the variation in several TLC R_f values depends on structural differences and the extent of ionization of the molecular constituents and, to a significant extent, the presence of intramolecular hydrogen bonding (24–26). Due to clear differences in R_f values and staining, the lipid spots were described separately. Finally, there is the possibility that *L. monocytogenes* contains polar hopanoid lipids (a class of pentacyclic triterpene lipids) as Moreau *et al.* (27) reported for several species of bacteria.

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Interference of Free Fatty Acids from the Hepatopancreas of Mussels with the Mouse Bioassay for Shellfish Toxins

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ABSTRACT: Determination of free fatty acids (FFA) in hepatopancreas of mussel, *Mytilus coruscus,* contaminated by diarrhetic shellfish poisoning (DSP) toxins, was carried out by high-performance liquid chromatography–fluorometry as their 9-anthryldiazomethane derivatives. The FFA in the hepatopancreas of mussels during May 30–June 14 were 443–666 μg/g. A marked increase in FFA content was observed on June 20 (2197 μg/g hepatopancreas) and June 27 (6322 μg/g hepatopancreas). The proportion of polyunsaturated fatty acids (PUFA), including 20:4n-6, 20:5n-3, and 22:6n-3, in mussel FFA increased during the experimental period. Diatoms almost completely dominated the phytoplankton community at the experimental site, suggesting that diatoms were the origin of the PUFA accumulated in mussels. The toxicity of FFA in mussel hepatopancreas on June 27 was sufficiently high as to interfere with the mouse bioassay by injection of an extract of shellfish, the official method for DSP toxin analysis.

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Diarrhetic shellfish poisoning (DSP) has become a well-known and worldwide problem since it was first reported by Yasumoto *et al.* (1,2). The monitoring of DSP toxin contents in shellfish is officially carried out by the mouse bioassay. Many commercially important shellfish are banned from harvesting during the toxicity period, causing serious economic damage to the shellfish industry. The accumulation of DSP toxins in shellfish is presumed to result from their ingestion of phytoplankton producing DSP toxins (3). However, the mouse bioassay for shellfish collected at a monitoring site at which DSP toxin-producing phytoplankton have not been observed occasionally shows positive results. The reason for this is partly because of the false positive results caused by free fatty acids (FFA) contained in shellfish; FFA show toxicity in the mouse bioassay (4–7).

In 1994, an incident of DSP occurred on the Niigata coast, Japan. Shellfish harvesting was prohibited from May 16 to August 2 (8). We determined the DSP toxins in mussels collected on the Niigata coast during the toxicity period by the mouse bioassay and by an instrumental method (5), and clarified that the contents of principal DSP toxins, okadaic acid (OA) and dinophysistoxin-1 (5), were too low to account for the mouse lethality of mussel extracts (8).

The present paper applies determination of FFA by high-performance liquid chromatography (HPLC)–fluorometry to elucidate the short-term changes in content and proportion of FFA in toxic mussel hepatopancreas collected on the Niigata coast during the DSP outbreak. The mouse lethality of FFA was also examined.

EXPERIMENTAL PROCEDURES

Sample material. Toxic mussels, *Mytilus coruscus,* were collected at a depth of 8 m at a monitoring station in the Niigata coastal area, Japan, from May 30 to June 27, 1994. Dates of the collection are shown in Table 1. The specimens selected were within a size range of 100–115 mm in shell length. The toxic hepatopancreas from five individual mussels were combined and used for each analysis.

One-liter sea-water samples for phytoplankton analyses also were collected at a depth of 8 m at the monitoring site with a Van Dorn sampler and fixed with formalin (5%). The counting and identification of phytoplankton was performed with a light microscope.

Extraction of FFA and derivatization. Extraction of FFA from mussel hepatopancreas was carried out immediately after sampling to avoid the production of FFA by exposing lipid to lipolytic enzymes during the storage of mussels. FFA in hepatopancreas of mussels were extracted with acetone by the procedures of the official mouse bioassay applied for DSP toxin analysis (9). About 10 g of the sample (combining the hepatopancreas of five individual mussels) was extracted in a homogenizer with 30 mL of acetone for two minutes at room temperature. After filtration, the residue was extracted two times with two 20-mL portions of acetone in a similar way. The combined acetone extract was made up to 100 mL by acetone. An aliquot (about 5 mL) of acetone extract corresponding to 0.5 g of hepatopancreas was evaporated to dryness under reduced pressure and dissolved in 2.5 mL of 80% methanol. FFA were extracted three times with 2.5 mL of *n*-hexane. The combined *n*-hexane extract was made up to 10

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Abbreviations: ADAM, 9-anthryldiazomethane; DSP, diarrhetic shellfish poisoning; FFA, free fatty acid; HPLC, high-performance liquid chromatography; MU, mouse unit; OA, okadaic acid; PUFA, polyunsaturated fatty acid.

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TABLE 1
Proportion (wt%) and Content of Free Fatty Acids in the Hepatopancreas of Mussels Determined by High-Performance Liquid Chromatography–Fluorometry

FFA[a]	RRT[b]	Date				
		May 30	June 6	June 14	June 20	June 27
Saturated						
14:0	0.73	5.1	4.7	6.2	3.8	2.9
16:0	0.85	27.5	21.4	20.8	20.5	19.9
18:0	1.00	13.7	9.4	8.9	6.4	6.2
20:0	1.20	9.6	7.9	2.5	1.7	0.4
Monounsaturated						
16:1n-7	0.73	11.4	12.6	13.5	12.1	9.6
18:1n-9	0.84	7.5	7.1	6.2	6.9	5.8
20:1n-9	0.98	6.9	7.3	5.6	7.0	8.1
Polyunsaturated						
18:2n-6	0.75	1.4	3.0	2.6	3.1	2.7
18:3n-3	0.65	ND[c]	ND	ND	ND	ND
18:4n-3	0.53	1.3	1.4	2.1	1.9	1.8
20:4n-6	0.70	1.4	2.5	2.3	2.8	4.5
20:5n-3	0.59	6.0	9.1	13.3	12.8	15.9
22:6n-3	0.64	8.2	13.6	16.0	21.0	22.2
Total		100.0	100.0	100.0	100.0	100.0
Total content in hepatopancreas (μg/g)		666	443	523	2197	6322
Water temperature[d] (°C)		17.0	17.8	17.9	16.2	17.7

[a]Free fatty acids. [b]Relative retention time with respect to 18:0. [c]Not detected. [d]At 8 m depth.

mL by *n*-hexane. An aliquot (25–250 μL) of the *n*-hexane extract was put in a colored vial and dried under nitrogen. The residue was esterified in 100 μL of 0.1% 9-anthryldiazomethane (ADAM) methanol solution for one hour in the dark at ambient temperature, and 10 μL of the solution was directly analyzed by HPLC (10).

HPLC. HPLC separation was carried out with a Hitachi (Tokyo, Japan) L-6200/L6000 gradient system equipped with a Develosil ODS-5 column containing octadecyl phase bonded to 5 μm silica gel particles (250 mm × 4.6 mm i.d.; Nomura Chemical, Seto, Japan) with a gradient of solvent A (acetonitrile/methanol/water, 8:1:1, by vol) and solvent B (methanol) at 20°C and a flowrate of 1.1 mL/min (10). The following linear gradient was used: segment 1, 100% A for 20 min; segment 2, initial conditions changed to 100% B over 30 min; segment 3, 100% B for 50 min. The peaks of fluorescent derivatives were monitored with a Hitachi F-1050 spectrofluorometer. The excitation and emission wavelengths were set at 365 and 412 nm, respectively. Eluate containing 14:0 and 16:1n-7 was collected from the outlet of the fluoromonitor and rechromatographed on a Capcell Pak CN SG120 column containing cyanopropyl phase bonded to 5 μm silica gel particles (250 × 4.6 mm i.d.; Shiseido, Tokyo, Japan) with acetonitrile/water (7:3, vol/vol) as the mobile phase at ambient temperature (11). The flowrate was kept at 1.1 mL/min. Excitation and emission wavelengths of the fluoromonitor were 365 and 412 nm, respectively.

Identification and quantification of the FFA peaks were carried out by comparing relative retention times (as listed in Table 1) and peak area, respectively, with those of known ADAM derivatives of FFA standards (10).

RESULTS AND DISCUSSION

HPLC separation. Figure 1 shows a representative chromatogram of ADAM derivatives of FFA extracted from hepatopancreas of mussels separated on the Develosil ODS-5 column. A good separation of the principal FFA, except for 14:0 and 16:1n-7 having the same relative retention time (0.73) (Table 1), was obtained. Linearity of the total content of FFA in the extract with increasing amount of extract reacted with ADAM was confirmed (Fig. 2). This indicates that the reaction of ADAM with FFA is quantitative and reproducible. Up to *ca.* 30 μg of FFA was quantitatively reacted with ADAM in 100 μL of 0.1% ADAM methanol solution. Resolution of 14:0 and 16:1n-7 acids collected from HPLC on the Develosil ODS-5 column was carried out by second HPLC run on the Capcell Pak CN SG120 column to calculate the proportion of these two FFA (11).

Short-term changes in proportion and content of FFA. Table 1 shows the proportion and content of FFA in hepatopancreas of mussels determined by HPLC–fluorometry. The major FFA were 16:0, 16:1n-7, 20:5n-3, and 22:6n-3. This is consistent with earlier observations on mussel lipids

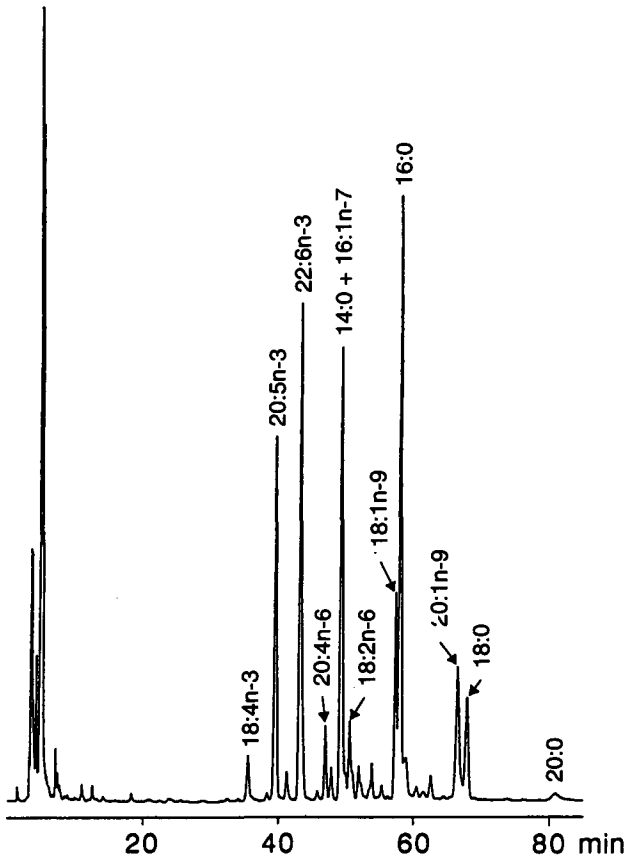


FIG. 1. High-performance liquid chromatography separation of 9-anthryldiazomethane derivatives of free fatty acids from the hepatopancreas of mussels collected at the Niigata coast (June 20) on the Develosil ODS-5 column (Nomura Chemical, Seto, Japan). An aliquot of the extract (1/800 of 1 g hepatopancreas of mussels) was injected. For derivatization procedure and chromatographic conditions, see text.

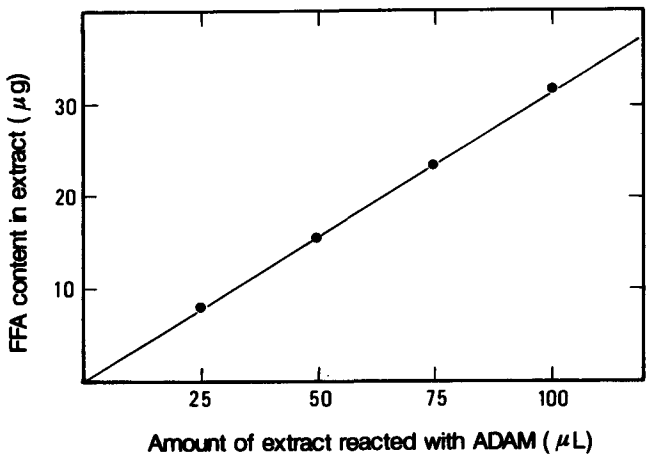


FIG. 2. Linearity of total content of free fatty acids (FFA) in extract (mussels, June 27) with increasing amount of extract reacted with 9-anthryldiazomethane (ADAM). An aliquot of the *n*-hexane extract was reacted with ADAM in 100 μL of 0.1% ADAM methanol solution. Extracts from hepatopancreas of mussels collected on June 27 were used.

(12–15). The total contents of FFA observed in hepatopancreas for the period from May 30 to June 14 were 443–666 μg/g. The values were higher than those in mussels collected in the Gulf of Trieste (136–360 μg/g) (16) and in scallops from Mutsu Bay (270 μg/g) (10). A marked increase in FFA contents in hepatopancreas of mussels was observed on June 20 and 27. The proportion of polyunsaturated fatty acids (PUFA), especially 20:4n-6, 20:5n-3, and 22:6n-3, increased together with the decrease in 14:0, 16:0, 18:0, and 20:0 acid percentages. Although the proportion of FFA does not directly reflect that of total lipids, an increase in the PUFA percentages in the FFA compositions indicates accumulation of these fatty acids in total lipids of mussels. Generally, the fatty acid composition of marine invertebrates is influenced by a host of environmental factors. The most important contributing factors to any variation in fatty acid composition are temperature and food availability (12,13,15). Since the water temperature where the mussels were cultured (8 m depth) did not change significantly (Table 1), an effect of temperature on the FFA composition in mussels was not considered. Because mussels are incapable of synthesizing n-3 and n-6 series PUFA (12), an increase of PUFA is likely to be a consequence of food abundance or changes in the intensity of feeding activity.

Table 2 shows the compositions and cell densities (cells/L) of phytoplankton present where the mussels were cultured. The most abundant plankton species were diatoms, *Leptocylindrus danicus* (May 30 and June 6), and *Chaetoceros* spp. (June 14 and 27). The total cell densities of phytoplankton did not change significantly (Table 2). Although the qualitative compositions of phytoplankton species observed at this site changed markedly, diatoms almost completely dominated the phytoplankton community. While there was 12.5% of dinoflagellates in the total phytoplankton population of June 27, a peak corresponding to 18:5n-3 which has been proposed as a biological marker for dinoflagellates in marine food-chain study (17) was not detected from mussel hepatopancreas collected on June 27. This suggests that the increases of both FFA contents and proportion of PUFA in mussels on June 27 are not related to dinoflagellates. These results suggest that PUFA accumulated in mussels are derived from diatoms because diatoms produce such PUFA (11,18–21). The FFA accumulated in hepatopancreas of mussels on June 20 and 27 may be intact or semi-intact algal cells because molluscs feed partly by phagocytosis.

Mouse lethality induced by FFA. The calculated toxicity of hepatopancreas of mussels assigned to FFA during the experimental period was on May 30 (0.02 MU/g), June 6 (0.02 MU/g), June 14 (0.02 MU/g), June 20 (0.11 MU/g), June 27 (0.37 MU/g). The mouse lethality for FFA was calculated from the respective FFA contents (Table 1) and the toxicity of standard FFA data reported previously (4). Generally, the mouse bioassay for DSP toxin analysis expresses toxicity by a mouse unit (MU), which is defined as the amount of a toxin, or toxins, to kill a male mouse of ddY strain of 20 g body weight in 24 h. Hepatopancreas having mouse lethalities

TABLE 2
Relative Phytoplankton Species Compositions (%) and Cell Densities (cells/L) at the Monitoring Station

Species	Date				
	May 30	June 6	June 14	June 20	June 27
Amphora spp.	11.8	—	—	57.8	—
Chaetoceros spp.	—	—	72.7	—	87.5
Leptocylindrus danicus	58.7	100.0	—	—	—
Navicula spp.	5.9	—	—	21.1	—
Nitzschia spp.	—	—	27.3	5.3	—
Skeletonema costatum	11.8	—	—	—	—
Others	11.8	—	—	15.8	—
Diatoms[a]	100.0	100.0	100.0	100.0	87.5
Dinoflagellates	—	—	—	—	12.5
Total (%)	100.0	100.0	100.0	100.0	100.0
Cell densities (cells/L)	4.3×10^5	5.5×10^5	3.0×10^5	4.8×10^5	4.0×10^5

[a]Total diatom percentages.

above 0.5 MU/g are regarded as toxic and unsatisfactory for food use (9). The toxicities of hepatopancreas used in the present study as determined by the mouse bioassay and by HPLC were 0.5–1.0 and 0.02–0.13 MU/g, respectively (8). The calculated toxicity from May 30 to June 14 was 0.02 MU/g. This value was far below that required for a quarantine level (0.5 MU/g) in shellfish hepatopancreas. A marked increase in the calculated toxicity was obtained on June 20 and 27. The calculated toxicity of FFA on June 27 was of a high enough value to conceivably give rise to a false-positive result in the official mouse bioassay for DSP toxins. Although the FFA content in the hepatopancreas of mussels on June 27 was 9.5 times higher than that on May 30 (Table 1), the calculated toxicity from FFA on June 27 was 18.5 times that on May 30. This discrepancy arises from the difference in toxicity of respective FFA; long-chain PUFA, especially 20:4n-6 and 20:5n-3, are agents active as mouse lethal material (4).

In the official mouse bioassay for DSP toxins, material extracted from shellfish is injected i.p. into mice. Generally, material injected i.p. is absorbed into the lymph system, and this drains into the circulating blood *via* two channels, the thoracic duct and the right lymphatic duct, respectively. The thoracic duct receives all materials from the abdomen. It is known that direct injection of 20:4n-6 acid into the blood vessels of animals is almost immediately lethal (22). Mouse lethality induced by i.p. injection of FFA suggests that the mice may have died as a result of sufficient FFA being rapidly passed into the circulating blood. FFA are formed in foods by lingual and gastric lipases, and the previous experiments in animals and humans fed PUFA as FFA does not refer to any toxicity assignable to these FFA (23–25). On the other hand, DSP toxin, such as OA, is a very potent inhibitor of protein phosphatase 1 and protein phosphatase 2A (26). It is suggested that OA causes diarrhea by stimulating the phosphorylation of the protein(s) that control sodium secretion by intestinal cells (26). Therefore, it is very important to recognize that FFA are not implicated directly in any way in the human exhibiting symptoms of DSP.

In conclusion, results of the present study demonstrate that the proportion and content of FFA in mussel hepatopancreas show considerable short-term changes. It is suggested that PUFA accumulated in mussels were derived from diatoms. The calculated toxicity of FFA in mussel hepatopancreas on June 27 demonstrates that there was a value sufficiently high to interfere with the mouse bioassay. The result suggests that the determination of FFA in mussels coupled with the official mouse bioassay for DSP toxin analysis is necessary for a more accurate interpretation of DSP toxicity.

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Unusually High Levels of Eicosatetraenoic, Eicosapentaenoic, and Docosahexaenoic Fatty Acids in Palestinian Freshwater Sponges

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ABSTRACT: The fatty acid composition of three freshwater sponges—*Ephydatia syriaca, Nudospongilla* sp., and *Cortispongilla barroisi*—were studied. Twenty principal fatty acids, and unusually high levels of eicosatetraenoic (5,8,11,14-20:4 up to 10.1% of the total acid mixture), eicosapentaenoic (5,8,11,14,17-20:5 up to 11.6%), and docosahexaenoic acids (4,7,10,13,16,19-22:6 up to 11.8%) were detected. The only demospongic acid found was 5,9,17-hexacosatrienoic acid (1.8–3.7%).

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At the present time, a definite opinion has been formed concerning the unique nature of sponge biomembranes (1). Sponges can be regarded as live fossils of the contemporary animal world due to the fact that they have undergone practically no evolutionary development, i.e., their present representatives probably remain at the evolutionary level of their remote precursors (2). Sponge lipids differ markedly in their chemical and biochemical properties from lipids obtained from other living organisms (1). Rare lipids, including fatty acids, have been identified in marine sponges, mostly long-chain fatty acids (C_{24}–C_{36}) of straight, branched, and brominated structure (3). Because these acids were isolated for the first time from marine sponges, they are often called "demospongic acids" (1). The total content of demospongic acids can reach up to 80% in some marine sponge species (3,4), and a major part of them is found in individual phospholipid classes (1,3). There is limited information on whether freshwater sponges contain demospongic acids (5–12).

In this work we report a study of the fatty acid composition of three freshwater sponges, which were collected in an expedition to the Jordan river, Lake Hula, and the Sea of Galilee, Palestine.

MATERIALS AND METHODS

Sampling and lipid analyses. The freshwater sponge *Ephydatia syriaca* was collected in August 1993, in the spring of the Jordan river (Banias, near Mountain Hermon); the freshwater sponge *Nudospongilla* sp. was collected in May 1994, in Lake Hula; and the freshwater sponge *Cortispongilla barroisi* was collected in July 1994, in the Sea of Galilee (Lake Tiberias, Lake Kinneret, Palestine). All sponges were washed in water, carefully cleaned of all debris, and cut into small pieces. Immediate lipid extraction after collection was done as described elsewhere (9).

Gas chromatographic/mass spectrometric analyses of fatty acid methyl esters. High-resolution mass spectrometry was done using a MAT 90 magnetic sector mass spectrometer (Finnigan, San Jose, CA) in the electron ionization mode. Gas chromatography/mass spectrometry of fatty acid mixture was done using a Finnegan 1020 B single-state quadrupole gas chromatography/mass spectrometry instrument in the electron ionization mode. Injection temperature (splitless injection) was 100°C, and a fused-silica capillary column (Supelcowax 10; 60 m × 0.25 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, PA) was used. The temperature program was as follows: 100°C for 1 min, than increased at 20°C/min to 180°C and at 2°C/min to 280°C, which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm s^{-1}. All spectra were scanned within the range *m/z* 70–450.

RESULTS AND DISCUSSION

The fatty acid compositions of three sponge species are presented in Table 1. Twenty principal fatty acids were detected in the sponge total lipids. All the principal fatty acids identified are "classical" acids which are found in invertebrates (13). Only one demospongic acid, namely 5,9,17-26:3 (from 1.8 to 3.7%) was found in all sponge species. Over the last two decades it has been established that the majority of marine sponges contain trace amounts of both 20:4/20:5 and/or 22:6 fatty acids (3,14–30). Both isomers of eicosatetraenoic acid were found in some freshwater sponges ranging in amount from 1.4 to 11.8% (5–7,10–12) and in marine sponges ranging from 0.2 to 18.3% (16,31–49) (see Table 2). Higher

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TABLE 1
Principal Fatty Acids of the Freshwater Sponge

Fatty acids	*Ephydatia syriaca*	*Nudospongilla* sp.	*Cortispongilla barroisi*
14:0	3.2	1.8	2.3
16:0	8.5	10.6	9.4
18:0	4.5	5.1	3.9
20:0	1.6	1.8	2.1
Saturated	17.8	19.3	17.7
7-16:1	1.1	1.2	1.5
9-16:1	5.5	6.2	7.3
9-18:1	7.7	8.9	9.4
11-18:1	2.1	2.2	2.1
11-20:1	1.4	1.4	1.9
11-22:1	1.5	1.6	1.5
Monoenoic	19.3	21.5	23.7
6,9-18:2	5.7	5.8	6.6
9,12-18:2	5.4	6.4	5.3
12,15-18:2	2.2	2.4	2.5
Dienoic	13.3	14.6	14.4
6,9,12-18:3	3.0	3.6	3.8
9,12,15-18:3	9.7	9.6	10.6
5,9,17-26:3	3.6	3.7	1.8
Trienoic	16.3	16.9	16.2
6,9,12,15-18:4	1.7	0.7	0.6
5,8,11,14-20:4	10.1	9.5	9.5
5,8,11,14,17-20:5	9.7	9.5	11.6
4,7,10,13,16,19-22:6	11.8	8.0	6.3
Sum	33.3	27.0	27.4

amounts were found in *Phakellia aruensis*, 12.5% (40); *Ectyoplasia ferox*, 14.1% (35); and in *Spheciospongi confoederata*, 18.3% (47).

Eicosapentaenoic acid was found in some marine sponges (see Table 2) and larger amounts were detected in freshwater sponges (up to 17.2% (10). Docosahexaenoic acid was the major lipid component in the marine sponge *Microciona prolifera*, varying from 15.4 to 25.4% of total lipids (43,45–47). Freshwater sponges usually contained this acid in amounts which ranged from 0.8 to 4.1% (Table 2). In the present study, unusually high levels of eicosatetraenoic, eicosapentaenoic, and docosahexaenoic acids were found in all freshwater sponges (Table 1). The percentage of total lipids formed by these three fatty acids as detected in *E. syriaca* was 31.5%; in *Nudospongilla* sp., 26.3%; and in *C. barroisi*, 26.8%. Weissmann *et al.* (43) reported that a high level of 22:6 fatty acid was found in two phospholipids of the marine sponge *M. prolifera*, namely in phosphatidylcholine (52%) and in phosphatidylinositol (29%). The authors indicated that the high level of 22:6 acid could be the result of differences in the local environment of the samples collected, and appeared to be related to a role for this fatty acid in stimulus–response coupling in these cells.

More interesting data could be associated with 5,9,17-26:3 fatty acid, which was found to be the major demospongic fatty acid in some Palestinian freshwater sponges. According to Hahn *et al.* (7) and Dembitsky *et al.* (9), who studied the fatty acid total content of the freshwater sponges *E. fluviatilis* and *Euspongilla lacustris*, only 5,9,19-26:3 acid was 14.9%

TABLE 2
Comparative Distribution of Eicosatetraenoic, Eicosapentaenoic and Docosahexaenoic Fatty Acids in Total Lipids of Some Freshwater and Marine Sponge Species (class Demospongiae)

Sponge species	Abundance (wt%) 20:4[a]	20:5[b]	22:6[c]	References
Freshwater species				
Baicalospoggia bacilifera	4.3	9.4	0.8	6
B. bacilifera	2.6	1.8	4.1	11
B. baicalensis	2.7	12.2	0.5	10
B. intermedia	5.9	7.1	4.1	6
B. intermedia	3.2	2.0	2.9	11
B. intermedia (green)	1.4	4.5	0.4	10
B. intermedia (grey)	1.3	5.7	0.3	10
Ephydatia fluviatilis	11.8	—	—	7
Euspongilla lacustris	3.7	—	—	9
Lubomirskia abietina	5.2	7.8	2.0	5
L. baicalensis	3.9	6.9	0.9	5
L. baicalensis	1.9	1.7	2.9	12
L. fusifera	3.5	6.0	1.2	5
Swartschewskia bacilifera	3.4	7.6	0.7	10
S. papyracea	4.6	10.1	3.0	6
S. papyracea	4.9	17.2	1.5	10
Marine species				
Adocia cinerea	3.9	6.9	4.6	31
Agelas sp.	3.3	—	—	32
Amphimedon viridis	3.5[c]	1.4[d]	0.7	49
Aplysia archeri	6.9	—	—	16
Chondrosia remiformis	3.0	—	—	34
Cornacuspongia gen. sp.	1.5	5.9	0.3	31
Dysidea fragilis	4.3	2.9	2.9	38
D. herbacea	—	2.0	1.0	40
Ectyoplasia ferox	14.1	—	—	35
Halichondria panicea	4.0	5.6	4.2	44
Homaxinella subdola	0.4	8.6	1.2	31
Hymeniacidon sanguinea	2.7	3.8	0.1	39
Hymeniacidonidae gen. sp.	9.5[d]	—	—	42
Microciona prolifera	4.7	—	25.4[e]	46
M. prolifera	6.2	—	18.9[f]	46
M. prolifera	2.0[d]	3.9[d]	22.4[d]	43
M. prolifera	4.3	—	15.4	47
M. prolifera	1.2	3.6	15.4	45
Mycale laevis	4.7	—	—	33
Myrmekioderma styx	2.0	—	—	34
Myxilla incrustans	3.8	13.3	5.9	31
Ophithaspongia pennata	3.9	1.5	0.2	31
Petrosia pellasarca	1.0	—	—	36
Petrosia sp.	3.3	—	1.8	37
Phakellia aruensis	12.5[g]	2.4[g]	5.4[g]	40
P. cribrosa	0.2	1.9	2.5	31
Phyllospongia papyracea	—	0.1	1.2	40
Pseudaxinyssa sp.	0.5	0.3	0.3	40
Rhopaloeides odorabile	0.5[g]	—	0.2[g]	40
Reniera sp.	—	—	6.0	48
Spheciospongi confoederata	18.3	—	—	47
Suberites domuncula	3.4	4.2	3.9	31
Tylodesma aff. rosca	3.4	4.8	4.5	31

[a]Two isomers, namely 5,8,11,14-20:4 and 6,9,12,15-20:4. [b]20:5 = 5,8,11,14,17-20:5. [c]22:6 = 4,7,10,13,16,19-22:6. [d]Total phospholipids. [e]Collected in July. [f]Collected in December. [g]Without cyanobacteria.

of the total fatty acid methyl esters (phosphatidylethanolamine, 19.8%; and phosphatidylserine, 14.8%) and 10.3% (phospholipid fraction, 20.9%), respectively, in these two species.

Previous gas chromatography/mass spectrometry analyses of the fatty acid levels in three freshwater sponges from Lake Baikal (11,12) indicated that 5,9,17-26:3 acid is the major demospongic fatty acid. Prior and current data show the principal biosynthetic sequence that seems to be operative in the freshwater sponge species. The biosynthetic pathway of the 5,9,17-26:3 acid is predominant.

The results presented in this work are of interest because they clearly demonstrate that three polyunsaturated fatty acids—eicosatetraenoic, eicosapentaenoic and docosahexaenoic—and the demospongic acid 5,9,17-26:3 are the principal fatty acids in freshwater sponges. Further work is in progress toward elucidating the structure of some unusual minor fatty acids.

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Lipidic Characterization of Full-Grown Amphibian Oocytes and Their Plasma Membrane-Enriched Fractions

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ABSTRACT: The content and composition of neutral lipids and phosphoglycerides from full-grown prophase-arrested *Bufo arenarum* Hensel oocytes and from their ghost preparations were studied. The ghosts obtained are highly enriched in plasma membrane as suggested by the activity of 5'-nucleotidase, a marker enzyme, and the level of typical membrane components such as sphingomyelin, phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidic acid. In whole oocytes, triacylglyceride (TAG) comprises about 60% of the total lipids followed by phosphatidylcholine (PC), cholesterol, and phosphatidylethanolamine (PE). TAG and diacylglycerides have a similar unsaturation index. PC and PE account for about 80% of the phosphoglycerides in the whole oocyte and in their plasma membrane-enriched fractions. Arachidonic acid (20:4n-6), 18:0, and 16:0 make up about 80 mol% of the total fatty acids in PI in whole oocytes and ghost fractions. The unsaturation index in PS is higher in intact oocytes than in ghost preparations, probably owing to the significant amount of 20:4n-6 which comprises 23 mol% of the total fatty acids in whole oocytes. The fatty acid profile in phosphatidic acid from whole oocytes is rather different from that in ghosts. Sphingomyelin contains mainly saturated and monounsaturated fatty acids, 24:1 being the principal very long chain unsaturated fatty acid in both oocytes and ghosts.
Lipids 31, 651–656 (1996).

Amphibian oocytes are larger and simpler than most mammalian cell types, a phenomenon which facilitates their use for electrophysiological and biochemical measurements. The full-grown amphibian oocyte system offers several advantages for investigating the responses of both endogenous receptors (1) and exogenous receptors which have been translated, processed, and rendered active from injected mammalian mRNA (2). The ability of agonist-receptor complexes to bind to effector molecules may be modulated by the head group and fatty acid composition of membrane lipids (3). Some phosphoglycerides and fatty acids are crucial for the activity of certain enzymes such as protein kinase C, adenylate cyclase, and guanylate cyclase (4–6). In addition, long-chain fatty acids applied to the cytoplasmic membrane surface of intact BC_3H-1 muscle cells triggered drastic changes in acetylcholine receptor gating properties (7). It is clear that lipid composition is involved in the modulation of nicotinic acetylcholine receptor function (8). There is growing evidence that physiological response to progesterone, the hormone that acts at the full-grown amphibian oocyte plasma membrane receptor to induce meiotic division, may be modulated by changes in the phosphoglyceride microenvironments within the plasma membrane (9).

A detailed knowledge of the native hydrophobic environment is required for the exploration of those specific features of lipids associated with the presence of oocyte receptors. An understanding of the membrane's composition is also a prerequisite to metabolic studies focused on the understanding of the possible involvement of lipids in the receptor functions. This paper is concerned with the content and fatty acid profile of neutral lipids and phosphoglycerides in whole *Bufo arenarum* Hensel oocytes arrested in the prophase of the first meiotic division. In addition, highly enriched plasma membrane fractions (ghosts) were obtained, and a characterization of their lipids was performed.

MATERIALS AND METHODS

Experimental system. Mature females of the toad *B. arenarum* Hensel were obtained commercially and maintained under laboratory conditions. The ovarian tissue was surgically removed and transferred to ND 96 solution (96 mM NaCl, 1 mM Mg Cl_2, 2 mM KCl, 5 mM HEPES, pH 7.4; Sigma, St. Louis, MO). After rinsing, it was cut into small pieces, and full-grown prophase-arrested oocytes, stage V according to Valdéz Toledo and Pisanó (10), were isolated under a stereotaxic microscope. Only those ovaries containing a large population of full-grown oocytes were used. Oocytes were treated with 0.25% (wt/vol) collagenase (Sigma Type I) in ND 96 solution with agitation for one hour at room temperature. After collagenase treatment, oocytes free from follicular cells were rinsed several times with ND 96 solution.

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Abbreviations: DAG, diacylglycerides; FFA, free fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; TAG, triacylglycerides; TLC, thin-layer chromatography; UI, unsaturation index.

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Ghost preparations. The methodology originally developed for obtaining ghosts (enriched-plasma membrane preparations) from sea urchin oocytes (11) was adapted. Oocytes were transferred to 10 vol of a solution containing 0.1 M $MgCl_2$, 0.01 M TRIS (Sigma), pH 7.5, and kept there for at least 30 min in order to stabilize the oocyte membrane and to wash the surface. The oocytes were sedimented at 3,000 rpm for 5 min and the supernatant discarded. Oocytes were osmotically lysed by resuspension in 0.01 M $MgCl_2$, 0.01 M TRIS, pH 8.0 for 30 min. Lysis was augmented by gently homogenizing with a Teflon pestle. Lysed cells were sedimented, and the resulting pellet was resuspended in the diluted $MgCl_2$ and then sedimented again. The pellet contained oocyte ghosts was applied on 40% sucrose solution and centrifuged at 3,000 rpm for 5 min. The purified ghosts were recovered from the interphase and washed with diluted $MgCl_2$ to eliminate sucrose.

Lipid analysis. Lipid extraction from whole oocytes and ghost preparations was performed following Folch *et al.* (12). Phosphoglycerides were separated by two-dimensional thin-layer chromatography (TLC) on silica gel H according to Rouser *et al.* (13). Neutral lipids were separated by monodimensional TLC on silica gel G using hexane/diethyl ether/acetic acid (40:60:1, by vol). Phosphoglycerides were located after exposure of the plates to I_2 vapors and quantitated by phosphorus analysis (13) after digestion with perchloric acid.

For determination of fatty acid composition, the lipids were separated by TLC as described above, and spots were visualized under ultraviolet light after spraying the plates with 2′-7′-dichlorofluorescein in methanol. Fatty acid methyl esters were prepared according to the method of Morrison and Smith (14) using boron trifluoride (14% in methanol; Sigma). Fatty acid analysis was performed by temperature-programmed gas–liquid chromatography using 21:0 methyl ester as internal standard. Two glass columns (2 m × 0.2 cm i.d.) packed with 15% OV-275 on 80-120 Chromosorb WAW (Supelco, Bellefonte, PA) were connected to two flame-ionization detectors operated in the dual-differential mode. The initial and final oven temperatures were 160 and 220°C, respectively, and the rate of increase was 5°C/min. Injector and detector temperatures were 220 and 230°C, respectively. The carrier gas was N_2 (30 mL/min). Chromatograms were quantitated with a CDS-111 Varian integrator (Palo Alto, CA). Peaks were identified by comparison of retention times with those of standards. Unsaturation index was determined as the sum of percentage of individual unsaturated fatty acids times number of double bonds.

During all the procedures (lipid extraction, solvent evaporation, TLC spotting, drying and spraying of TLC plates, and the described derivatization), the lipids were kept in an N_2 atmosphere. All organic solvents were of analytical grade. Cholesterol spots were scraped from TLC plates, eluted with chloroform, and quantitated using a commercially available nonenzymatic assay (Wiener Laboratories, Rosario, Argentina). In this procedure, cholesterol was subjected to oxidation with ferric ions in sulfuric medium. The absorbance of the formed red chromogen was measured spectrophotometrically at 560 nm.

Other analytical methods. Proteins were determined by Lowry *et al.* (15) after extraction with 1 N NaOH using crystalline bovine serum albumin as standard. The activity of 5′ nucleotidase as an enzyme marker of plasma membrane was measured according to Michell and Hawthorne (16). Statistical analysis was performed using Student's *t*-test with the values representing the mean standard deviation of the total number of samples indicated in each legend.

RESULTS AND DISCUSSION

The results of this study provide the first detailed lipid analysis of full-grown prophase-arrested amphibian oocytes. The content and composition of neutral and polar lipids of *B. arenarum* full-grown oocytes and their plasma membrane-enriched fractions (ghosts) were analyzed.

Although there is evidence to suggest that physiological responses to agonists may involve changes in the lipid microenvironment within the plasma membrane, to date there are no biochemical analyses of the lipid composition of amphibian oocyte plasma membrane. In order to analyze lipid composition, a procedure to obtain ghosts (Fig. 1) was adapted to permit the preparation of milligram quantities of plasma membrane. Typically, 15 mg of plasma membrane protein was obtained from 300–400 mg protein of oocytes. The activity of 5′-nucleotidase, a plasma membrane marker enzyme, was about 17-fold higher in ghosts than in oocyte homogenates, suggesting a significant enrichment in plasma membrane in these preparations (data not shown). This finding is consistent with the net increases in the amount of typical plasma membrane phosphoglycerides such as phosphatidic acid, phosphatidylserine (PS), phosphatidylinositol (PI), and sphingomyelin (SM) (Table 1). The percentage of phosphatidic acid in whole oocytes was 0.1, whereas in ghost preparations it was 1.3. The respective values for PS and PI ranged between 1.0 and 2.9 and 3.4 to 5.4, respectively, when whole oocytes and ghost preparations were compared with-

TABLE 1
***Lipid Composition of Whole Full-Grown Prophase-Arrested Ooctyes and of Their Plasma Membrane-Enriched Fractions*[a]**

	Whole oocytes	Ghosts
Triacylglycerides	144.17 ± 0.19	—
Diacylglycerides	1.87 ± 0.17	1.77 ± 0.49
Free fatty acids	0.83 ± 0.19	9.24 ± 0.84
Cholesterol	20.12 ± 2.20	9.90 ± 0.25
Phosphatidic acid	0.09 ± 0.01	1.69 ± 0.31
Phosphatidylserine	0.93 ± 0.08	3.80 ± 0.15
Phosphatidylinositol	3.19 ± 0.22	7.03 ± 0.75
Sphingomyelin	7.93 ± 0.23	9.82 ± 0.58
Phosphatidylcholine	41.33 ± 6.02	63.74 ± 5.11
Phosphatidylethanolamine	18.78 ± 2.06	23.23 ± 1.82

[a]Lipids were isolated and quantitated as described in the Materials and Methods section. Results are presented as nmol of lipids per mg protein and are mean values ± SD from five independent samples.



FIG. 1. Ghosts of *Bufo arenarum* oocytes in stage V of oogenesis according to Valdéz Toledo and Pisanó (Ref. 10). Ghosts were obtained as described in the Materials and Methods section (20×).

out considering triacylglyceride (TAG) amounts which was not present in ghost preparations. SM content in ghost fractions was 1.2-fold higher than in whole oocytes.

In whole oocytes, TAG represented 60% of the total lipid amount followed by phosphatidylcholine (PC) (17%), cholesterol (8.5%), and phosphatidylethanolamine (PE) (7.9%). Neutral lipid content in whole oocytes was 2.3-fold higher than that of phosphoglycerides. In ghost preparations, PC comprised 49% of the total lipid content followed by PE (18%) and cholesterol (7.6%) (Table 1). In contrast to the case of whole oocytes, total phosphoglyceride content in ghosts was 5-fold higher than the level of neutral lipids. Identical results were obtained when phosphoglycerides were measured by phospholipidic phosphorus determination (data not shown) or by gas–liquid chromatography.

With regard to neutral lipid content in whole full-grown oocytes, TAG made up 86% of the total amount followed by cholesterol (12%), diacylglycerides (DAG) (1.1%), and free fatty acids (FFA) (0.5%). The high content of TAG is consistent with the putative employment of these neutral lipids in supplying the energy required after fertilization since there was a net fall in their content at this time. Fatty acids originating in TAG were rapidly utilized in other metabolic reactions (17,18). In coincidence, neutral lipids comprised most of the label when *B. arenarum* and *Xenopus laevis* full-grown oocytes were incubated with (^{3}H)-glycerol evidencing capacity to biosynthesize their own lipids (19).

When phosphoglyceride content was analyzed, it was found that in whole oocytes and ghost preparations the major constituents were PC and PE, followed by SM and PI (Table 1). In full-grown oocytes, SM and PC comprised 67% of the total content. In many systems, the sum of the two choline lipids constituted about half of the total phosphoglycerides, although the ratio of the amounts of these two components varied greatly (20). In *X. laevis* full-grown prophase-arrested oocytes, PC and PE comprised about 89% of the total phosphoglyceride amount. *Bufo arenarum* oocytes showed a lower amount of PE and a higher PC level than those of *X. laevis*. Other phosphoglycerides such as phosphatidic acid, PI, and SM were present in similar amounts in the two amphibian species (19). The fatty acid composition of neutral lipids in whole full-grown oocytes and ghosts is depicted in Table 2. Oleic (18:1), 16:0, 18:2n-6, and 16:1 were the principal fatty acids in TAG (about 89 mol% of the total fatty acid content) in whole oocytes. The 20:4n-6 was the major long-chain (C_{20}–C_{22}) polyenoic fatty acid. In DAG, 18:1, 16:0, and 18:0 amounted to 66 mol% of the total fatty acids. TAG and DAG showed a similar unsaturation index (UI) (about 113). The 18:1 was the principal FFA followed by 16:0, 18:2n-6, and 16:1. The 18:1 comprised about 30 mol% of the total fatty acids in all the neutral lipid classes analyzed. The high levels of 16:0, 18:1, and 18:2n-6 found in oocyte neutral lipids might serve as a storage pool for metabolic precursors, since these fatty acids are required as substrates for fatty acid elongation and desaturation or they may be important as a form of fuel, *via* β-oxidation.

In ghosts (Table 2), the major fatty acid in DAG was 16:0. Other quantitatively important fatty acids were 18:1 and 18:0. The 16:0, 18:1, and 18:0 comprised about 74 mol% of the total FFA. The FFA UI (70) was higher than that of DAG (49). Polyunsaturated fatty acids showed low values (about 10 mol%) in both lipid classes.

The acyl group distribution of PC, PE, and PI in full-grown oocytes and ghosts is shown in Table 3. The 16:0, 18:1, 18:2n-6, and 16:1 comprised 86 mol% of the total fatty acids of PC in whole oocytes. Fatty acids with carbon chain lengths between 20 and 22 represented 6 mol% in the total acyl group distribution, 20:4n-6 being the principal one. The acyl group distribution in PC was similar in full-grown *B. arenarum* and *X. laevis* oocytes (19). In PE, 18:1, 18:0, and 16:0 were the major fatty acids. There was a significant amount of 20:4n-6 in this phosphoglyceride. PE with 20:4n-6 at the 2-position was most abundant among the molecular species in the unfertilized *Rana nigromaculata* eggs (21). In full-grown *X. laevis* oocytes, the UI of PE was higher than in *B. arenarum,* owing to the contribution of 20:4n-6 and 22:6n-3 (19). The compositional analysis of PI in whole full-grown *B. arenarum*

TABLE 2
Fatty Acid Composition of Neutral Lipids in Whole Full-Grown Oocytes and Ghost Preparations[a]

Fatty acids	Whole oocytes			Ghosts	
	TAG	DAG	FFA	DAG	FFA
16:0	23.42 ± 2.12	22.44 ± 1.15	26.57 ± 4.10	41.42 ± 2.35	34.51 ± 1.72
16:1	14.41 ± 2.02	9.35 ± 0.21	10.66 ± 1.08	9.56 ± 0.92	2.38 ± 0.29
18:0	2.88 ± 0.29	17.88 ± 1.07	10.17 ± 1.47	19.19 ± 1.74	12.71 ± 1.28
18:1	31.01 ± 0.78	25.77 ± 0.98	29.44 ± 2.26	19.43 ± 1.25	26.42 ± 2.74
18:2n-6	20.23 ± 2.33	9.41 ± 1.38	13.09 ± 0.30	5.90 ± 0.09	9.28 ± 1.07
18:3n-3	4.51 ± 0.39	1.79 ± 0.09	3.30 ± 0.33	—	—
20:3n-6	—	7.64 ± 1.27	—	—	—
20:4n-6	1.89 ± 0.45	1.56 ± 0.09	3.21 ± 0.28	2.01 ± 0.03	3.44 ± 0.90
22:4n-6	0.17 ± 0.05	0.42 ± 0.12	1.26 ± 0.34	—	—
22:5n-6	0.75 ± 0.05	1.44 ± 0.17	1.27 ± 0.17	—	—
22:6n-3	0.75 ± 0.05	2.30 ± 0.17	1.02 ± 0.26	—	—
SFA	26.29 ± 2.55	40.32 ± 1.72	36.74 ± 4.08	63.52 ± 2.75	49.60 ± 2.73
MUFA	45.42 ± 1.23	35.12 ± 1.25	44.11 ± 2.92	29.00 ± 2.10	37.60 ± 3.20
PUFA	28.29 ± 0.32	24.56 ± 0.24	23.15 ± 0.38	7.90 ± 1.20	12.72 ± 1.92
UI	115.86 ± 2.06	111.15 ± 1.97	106.55 ± 1.18	48.82 ± 2.82	70.00 ± 2.35

[a]Results are presented as mean values ± SD (mol%) from five independent samples; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; UI: unsaturation index; TAG: triacylglycerides; DAG: diacylglycerides; FFA: free fatty acids.

oocytes indicates that 20:4n-6 constituted the major fatty acid representing 40 mol% of the total acyl groups (Table 3). Unfertilized eggs from *R. nigromaculata* and *B. arenarum* also were found to contain a large percentage of 20:4n-6 (22,23). Other important fatty acids in PI from full-grown *B. arenarum* oocytes were 18:0 and 16:0.

In ghosts, 16:0 and 18:1 were the principal fatty acids in PC followed by 18:2n-6 and 16:1, comprising altogether about 88 mol% of the total fatty acid amount. The 18:1, 16:0, and 18:0 were the principal acyl groups in PE. The UI was higher in PE than in PC. The 18:0 was the principal component in PI followed by 20:4n-6 and 16:0. The UI of PI was higher than that of PE and PC. PI from whole oocytes was more unsaturated than PI from ghost preparations.

The composition of PS, phosphatidic acid, and SM in whole oocytes and in ghost preparations is shown in Table 4. The UI in PS was higher in whole oocytes than in ghost preparations, owing probably to the marked content of 20:4n-6 and 22:6n-3. According to reports on other tissues, PS in whole oocytes denoted the highest UI among all lipids analyzed. The

TABLE 3
Fatty Acid Composition of Phosphatidylcholine, Phosphatidylethanolamine, and Phosphatidylinositol from Full-Grown Prophase-Arrested Oocytes and Ghost Preparations[a]

Fatty acids	Whole oocytes			Ghosts		
	PC	PE	PI	PC	PE	PI
14:0	1.42 ± 0.02	3.92 ± 0.31	—	—	—	—
16:0	32.56 ± 2.13	13.58 ± 0.58	16.03 ± 2.38	32.59 ± 2.04	14.41 ± 0.31	23.74 ± 2.60
16:1	9.60 ± 0.48	4.12 ± 0.42	1.94 ± 0.09	9.58 ± 0.84	2.71 ± 0.50	1.50 ± 0.20
18:0	5.76 ± 0.13	15.70 ± 0.84	26.98 ± 1.29	5.02 ± 0.48	13.58 ± 1.39	28.70 ± 0.63
18:1	29.48 ± 0.94	38.26 ± 1.77	9.05 ± 0.14	32.53 ± 1.34	43.85 ± 0.49	12.78 ± 0.21
18:2n-6	14.79 ± 1.07	7.88 ± 1.93	3.36 ± 0.05	13.35 ± 1.24	9.62 ± 0.58	0.86 ± 0.11
18:3n-3	—	—	—	1.37 ± 0.17	—	—
20:4n-6	3.59 ± 0.61	9.76 ± 0.67	40.07 ± 2.65	3.03 ± 0.60	7.45 ± 0.35	25.77 ± 2.32
20:5n-3	0.78 ± 0.10	2.30 ± 0.30	0.45 ± 0.04	0.13 ± 0.01	2.19 ± 0.36	0.54 ± 0.09
22:4n-6	—	0.37 ± 0.11	0.41 ± 0.12	0.20 ± 0.03	2.37 ± 0.16	0.86 ± 0.07
22:5n-6	0.20 ± 0.05	—	0.82 ± 0.03	0.86 ± 0.15	0.69 ± 0.12	2.59 ± 0.10
22:5n-3	1.31 ± 0.15	2.45 ± 0.31	0.08 ± 0.01	0.17 ± 0.04	1.11 ± 0.35	0.37 ± 0.01
22:6n-3	0.53 ± 0.04	1.66 ± 0.52	0.83 ± 0.16	0.47 ± 0.07	2.02 ± 0.63	2.29 ± 0.15
SFA	39.72 ± 0.89	33.20 ± 0.95	43.00 ± 1.09	37.61 ± 0.98	27.99 ± 0.65	52.43 ± 1.34
MUFA	39.08 ± 0.91	42.38 ± 1.05	10.98 ± 0.80	42.48 ± 1.23	46.56 ± 0.96	14.29 ± 0.98
PUFA	21.20 ± 0.98	24.43 ± 0.87	46.02 ± 2.18	18.81 ± 1.34	25.45 ± 1.03	33.58 ± 1.48
UI	97.62 ± 1.34	123.39 ± 2.13	191.33 ± 3.23	94.50 ± 1.56	137.14 ± 2.38	153.77 ± 2.31

[a]Data are mean values ± SD (mol%) of five independent samples; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI: phosphatidylinositol. Other abbreviations as in Table 2.

TABLE 4
Fatty Acid Composition of Phosphatidylserine, Phosphatidic Acid, and Sphingomyelin from Full-Grown Oocytes and Ghost Preparations[a]

Fatty acids	Phosphatidylserine		Phosphatidic acid		Fatty acids	Sphingomyelin	
	Whole oocytes	Ghosts	Whole oocytes	Ghosts		Whole oocytes	Ghosts
14:0	—	—	—	16.04 ± 0.59			
15:0	—	—	—	10.33 ± 1.43			
16:0	8.10 ± 0.31	12.61 ± 2.03	26.93 ± 0.54	13.77 ± 0.58	16:0	52.26 ± 0.97	34.35 ± 1.23
16:1	1.82 ± 0.29	3.05 ± 0.22	7.25 ± 0.64	7.70 ± 1.07	16:1	1.81 ± 0.13	1.81 ± 0.02
18:0	39.10 ± 3.41	33.85 ± 0.48	13.92 ± 2.34	8.87 ± 1.15	18:0	2.08 ± 0.12	3.40 ± 0.09
18:1	9.16 ± 1.15	16.70 ± 1.07	23.66 ± 0.84	8.09 ± 0.07	18:1	2.63 ± 0.09	4.45 ± 0.23
18:2n-6	1.29 ± 0.44	8.75 ± 0.14	9.41 ± 1.19	5.05 ± 0.11	18:2	1.12 ± 0.03	0.84 ± 0.02
20:3n-6	0.63 ± 0.04	—	4.76 ± 0.15	6.20 ± 0.79	20:1	1.62 ± 0.12	1.49 ± 0.15
20:4n-6	23.02 ± 6.12	9.21 ± 0.58	2.21 ± 0.25	5.45 ± 0.86	22:0	12.45 ± 0.94	11.81 ± 2.10
20:5n-3	1.37 ± 0.03	2.38 ± 0.18	1.09 ± 0.35	6.27 ± 0.96	22:1	4.06 ± 0.10	5.31 ± 0.72
22:4n-6	1.03 ± 0.03	5.26 ± 1.21	6.10 ± 0.79	—	24:0	6.97 ± 0.69	8.60 ± 1.03
22:5n-6	1.22 ± 0.15	4.26 ± 0.58	1.00 ± 0.17	5.25 ± 0.54	24:1	10.22 ± 0.34	12.77 ± 1.05
22:5n-3	2.57 ± 0.12	0.69 ± 0.01	3.66 ± 0.11	1.44 ± 0.15	25:0	2.79 ± 0.12	6.66 ± 0.72
22:6n-3	10.70 ± 3.10	3.24 ± 0.51	—	5.55 ± 1.01	26:0	1.79 ± 0.08	8.51 ± 0.62
SFA	47.21 ± 2.34	46.47 ± 1.98	40.86 ± 2.78	49.01 ± 1.49	SFA	78.54 ± 0.85	73.33 ± 1.92
MUFA	10.98 ± 0.97	19.75 ± 0.96	30.91 ± 1.76	15.79 ± 0.95	MUFA	20.34 ± 0.12	25.83 ± 0.15
PUFA	41.82 ± 1.26	33.78 ± 2.22	33.70 ± 1.41	35.21 ± 2.86	PUFA	1.12 ± 0.03	0.84 ± 0.02
UI	201.60 ± 5.67	151.18 ± 3.45	132.45 ± 5.89	159.13 ± 4.97	UI	22.58 ± 0.85	27.51 ± 0.52

[a]Results are presented as mean values ± SD (mol%) from five independent samples. Abbreviations as in Table 2.

fatty acid distribution in phosphatidic acid from whole oocytes was rather different from that in ghost preparations. The presence of 22:4n-6 in whole oocytes is of interest since this fatty acid is not widely prevalent in other cells.

SM contained mainly saturated and monounsaturated fatty acids. The 16:0 was the major saturated fatty acid in both whole oocytes and ghosts. Other quantitatively important saturated fatty acids were 22:0 and 24:0. The major very long chain unsaturated fatty acid was 24:1 which occurred in similar proportions in the two cases analyzed. SM showed the highest saturated fatty acid content (about 80 mol%) among all lipids analyzed.

Many vital cell functions are intimately dependent on the type and extent of polyunsaturation present in the membrane (24). The high unsaturation degrees of the whole oocyte PI and PS suggest that the new membranes to be formed after fertilization may require a degree of unsaturation higher than that required by the oocyte cell membrane itself. During *B. arenarum* early embryonic development, there is an active phosphoglyceride synthesis (18), but probably it is not enough to support the period of cell division. Amphibian eggs may be not only supplied with TAG reserves but also with phosphoglyceride reserves.

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An Improved Ultracentrifugation Method for the Separation of Cholesterol Carriers in Bile

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ABSTRACT: Vesicles and micelles, the major carriers of cholesterol in bile, play a role in the formation of cholesterol gallstones. A simple and rapid ultracentrifugation method was developed to isolate these biliary cholesterol carriers when only microliter amounts of bile were available. The proposed method employs a 46 to 0% sucrose density gradient, a NVT90 near-vertical rotor, and a centrifugation time of one hour. As little as 25 μL of bile can be used with no disruption of the carriers. The method was validated by comparison with gel filtration column chromatography using 6 mM taurocholate in the elution buffer. The sucrose linear density gradient ultracentrifugation procedure described here is simple, fast, and compares favorably with the gel filtration chromatography method for the separation of cholesterol carriers from bile.

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Cholesterol is solubilized in bile in unilamellar and multilamellar vesicles, as well as in simple and mixed micelles (1,2). It has been shown that patients with gallstones usually have a higher proportion of vesicles in their bile compared to control subjects without gallstones (3,4). It is believed that cholesterol crystals nucleate from cholesterol-rich multilamellar vesicles (5–8). Two analytical methods have been used to quantitate these cholesterol carriers in bile—gel filtration column chromatography (9–11) and density gradient ultracentrifugation (4,12–15). The amount of cholesterol in each carrier is difficult to determine as each of these methods has inherent analytical limitations. Gel filtration requires 100–200 μL of bile, exogenous bile salts in the elution buffer to maintain the integrity of the micelles, and a fractionation time of approximately 3 h (7,11,16–19). The ultracentrifugation method does not require added bile salts; however, artifacts may develop due to the effects of centrifugal force on the cholesterol carriers in the tube, the high hydrostatic pressure within the medium, and dilution during long run times (12,20). Run times as long as 18 h, required when using the swinging bucket rotor, may lead to the conversion of a proportion of the micelles to vesicles (15). Gradient formation using metrizamide, which was dissolved directly into bile, has been used to reduce the dilution factor (12,13). However, this method requires relatively large quantities of bile (about 5 mL).

Our laboratory has used a hamster model to study cholesterol gallstone disease in relationship to the distribution of the cholesterol carriers in bile (9,21). The hamster has, on average, 50 μL of bile in the gallbladder; no good estimate for the percentage distribution of vesicular cholesterol in bile is available when only microliter amounts of bile are present. Sömjen *et al.* (14; G. Sömjen, personal communication) used sucrose density gradient ultracentrifugation to separate the carriers in as little as 200 μL of bile. This method used a swinging bucket rotor and a centrifugation time of 18 h; this time period tended to alter the distribution of the cholesterol among its carriers. Vertical and near-vertical rotors can be used to obtain separations similar to those of the traditional swinging bucket rotor but with significantly reduced run times (usually 1–2 h). Vertical rotors have a shorter pathlength, resulting in greater efficiency and faster separations; therefore, there is a reduction in the centrifugal forces and the hydrostatic pressure on the medium that may result in artifacts (20,22).

The purpose of this study was to develop a rapid microanalytical ultracentrifugation method to separate the vesicles and micelles from hamster and model biles. We separated the cholesterol carriers in as little as 25 μL of bile using a sucrose density gradient in a near-vertical rotor with a run time of only 1 h. This new ultracentrifugation method gave similar results to gel filtration column chromatography (9).

MATERIALS AND METHODS

Bile samples. Fresh gallbladder bile was obtained from hamsters fed either a lithogenic or chow diet (23,24). Model bile (53.9% sodium taurocholate, 24.7% egg yolk lecithin, and 21.4% cholesterol, 3.7 g/dL total lipids; Sigma, St. Louis, MO) was made according to previously published methods (11). Bile was centrifuged at 5,000 rpm for 30 min. The supernatant was used immediately for separating the cholesterol carriers by either gel filtration chromatography or sucrose density gradient ultracentrifugation. A toluene solution of 0.12 μCi

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Abbreviation: CSI, Cholesterol saturation index.

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[1α,2α(n)-^{3}H]cholesterol and a chloroform/methanol (1:1) solution of 0.075 μCi 1-palmitoyl-2-[1-^{14}C]oleoyl phosphatidylcholine (Amersham Life Science, Arlington Heights, IL) were mixed and evaporated under N_2. Bile was added to the radioactive compounds and incubated for 1 h at 37°C with shaking to label the biliary cholesterol carriers.

Gel filtration column chromatography. The cholesterol carriers in labelled bile were separated on Sepharose CL-4B-200 with 6 mM taurocholate in the elution buffer using a modified procedure of Pattinson *et al.* (9,21,25,26).

Sucrose gradient ultracentrifugation: formation of sucrose gradient (Sömjen, G., personal communication). Sucrose was dissolved in 25–50 μL of bile (772 mg/mL bile) and placed at the bottom of a 5 mL centrifuge tube. A 46 to 0% (wt/vol) sucrose gradient containing 50 mM Trizma Hydrochloride (pH 8.0) and 1.5 mM EDTA (Sigma Chemical Co.) was carefully layered on top of the bile sample using a Jule J5 Gradient Former (Jule Inc., Biotechnology Division, New Haven, CT).

Rotors. OptiSeal polyallomer centrifuge tubes (#362185, 1/2 × 2 in., 4.9 mL; Beckman Instruments, Palo Alto, CA) containing the samples were spun at 50,000 rpm for either 1/2, 1, or 2 h in a titanium NVT90 near-vertical rotor (Beckman Instruments).

For comparison, some samples were placed in polyallomer centrifuge tubes (#326819, 1/2 × 2 in.; Beckman) and spun in an AH-650 swinging bucket rotor (DuPont Instruments/Sorvall, Wilmington, DE) at 40,000 rpm for 18 h at room temperature (Sömjen, G., personal communication). A Beckman Optima L-60 ultracentrifuge was used with both rotors.

Collection of fractions. Fractions were collected using a Beckman Fraction Recovery System. Recovery was made from the bottom of the puncturable polyallomer tubes with either needle puncture from the bottom of the tube or by putting a thin tube from the top to the bottom. The gradient was dispensed by an infusion pump equipped with an air-filled 25 mL disposable syringe. Twenty-five to 30 fractions were collected using a Pharmacia LKB FRAC-100 fraction collector (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) equipped with a drop counter (10 drops/fraction, 150–200 μL). An aliquot (20 μL) of each fraction was counted in a Beckman LS 3801 liquid scintillation system with automatic quench correction, using Aquasol-2 (NEN Products, Boston, MA) as the scintillation fluid.

Calculations and statistics. The percentage cholesterol in vesicles was determined using PeakFit v3.0, a nonlinear curve-fitting software from Jandel Scientific (San Rafael, CA). Data are reported as mean ± SD.

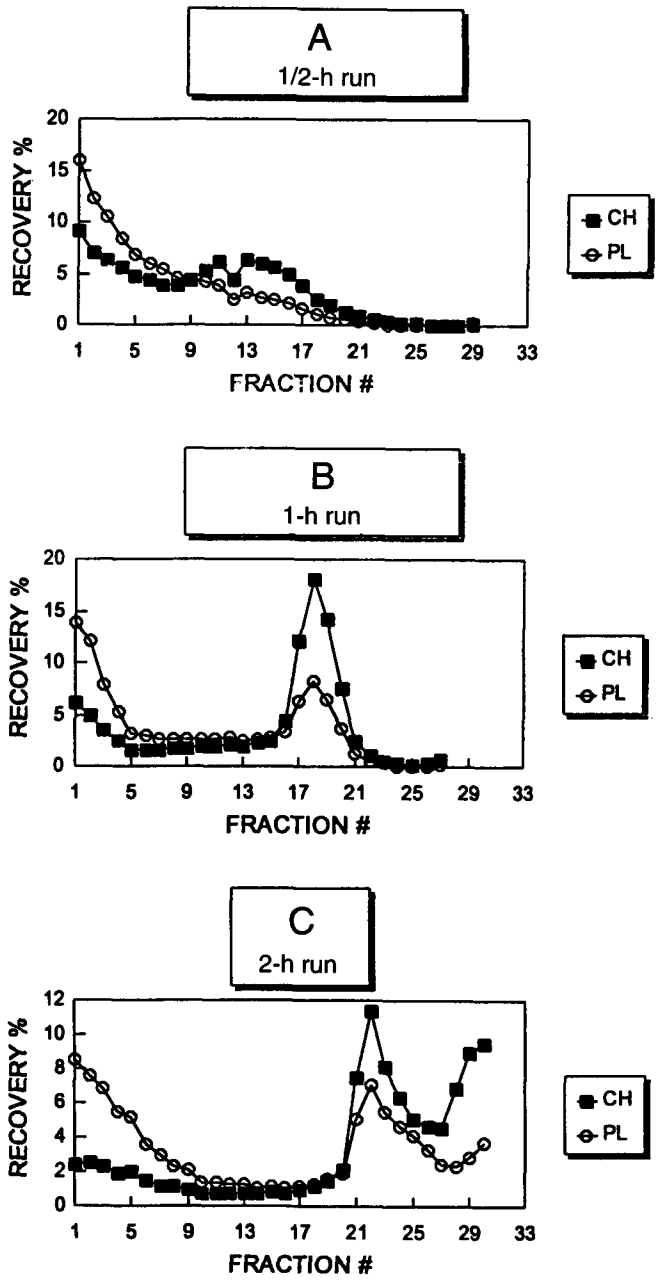


FIG. 1. Sucrose gradient ultracentrifugation (46 to 0%) of saturated model bile (cholesterol saturation index 309%) in an NVT90 near-vertical rotor (Beckman Instruments, Palo Alto, CA) spinning at 50,000 rpm for (A) 1/2 h, (B) 1 h, and (C) 2 h. Fractions were collected from the bottom of the tubes (fraction 1). See the Materials and Methods section for details of the ultracentrifugation procedure; CH, cholesterol; PL, phospholipid.

RESULTS AND DISCUSSION

The cholesterol carriers were separated from saturated and unsaturated model and hamster biles. Figure 1 compares run times for the sucrose gradient ultracentrifugation method in a near-vertical rotor using supersaturated model bile [cholesterol saturation index (CSI) 309%]. Two distinct peaks (micelles, fractions 1–6; vesicles, fractions 15–22) were detected using a run time of 1 h (Fig. 1B). A 1/2-h run was insufficient to separate the micelles from the vesicles (Fig. 1A); there was considerable overlap of the carriers as the vesicles did not fully migrate to the top of the tube. A 2-h run resulted in a micelle peak which was broad and tailed into the vesicle region (Fig. 1C). In addition, the vesicle region was now separated into two distinct peaks. The amount of cholesterol in the vesi-

TABLE 1.
Cholesterol (%) in Vesicles from Hamster Gallbladder Bile by Two Methods[a]

Bile sample	BA (%)	PL (%)	CH (%)	Total lipid g/dL	CSI (%)	% CH in vesicles	
						Column (n = 1)	Centrifuge (n = 4)
a	80.4	16.6	3.0	5.8	57	0	8 ± 4
b	74.1	19.2	6.6	8.5	104	42	44 ± 4
c	73.7	19.0	7.4	9.4	114	50	46 ± 4

[a]Sucrose gradient ultracentrifugation (46 to 0%) at 50,000 rpm in a near-vertical rotor for 1 h and gel filtration column chromatography with 6 mM taurocholate (TCA) in the elution buffer. See the Materials and Methods section for details of the two analytical methods. Values are mean ± SD; BA, bile acid; PL, phospholipids; CH, cholesterol; CSI, cholesterol saturation index.

cle region was 65% after 1/2 h, 78% after 1 h, and 80% after 2 h (Fig. 1). Thus, spinning the bile sample for either 1 or 2 h gave the same percentage cholesterol in vesicles. Nonlithogenic bile (CSI 55%) was separated into its carriers in the same manner. As expected, only one peak (fractions 1–7) was present after both 1 and 2 h, with some broadening after 2 h. Therefore, the micelle integrity was preserved under the ultracentrifugation conditions employed. Clearly, dilution was not a factor as cholesterol carriers were not detected in the vesicle region (figure not shown).

For comparison, we analyzed the cholesterol carriers in nonlithogenic bile (CSI 55%) using a swinging bucket rotor for 18 h. The micellar integrity was not preserved, about 40–50% of the cholesterol–phospholipid components were detected in the vesicular region. This artifact could have resulted from a longer run time for the tubes in a swinging bucket rotor as opposed to the near-vertical rotor (22). The carriers in the gradient were subjected to prolonged forces and high hydrostatic pressures, and their integrity was not maintained (20,22). Such artifacts do not seem to be a factor in the sucrose gradient ultracentrifugation method when runtimes are short (1–2 h).

The micelle and vesicle fractions isolated by ultracentrifugation were subsequently chromatographed by gel filtration using 6 mM taurocholate in the elution buffer. Vesicles and micelles were eluted from the column as expected, with no interconversion of one carrier to another. This result showed that (i) the ultracentrifugation method adequately separated the carriers and (ii) the integrity of the carriers was maintained on the gel filtration column using the 6 mM taurocholate elution buffer. If the bile salt concentration used in the elution buffer for gel filtration column was too low, micelles would be converted to vesicles; if the concentration was too high, the vesicles would be transformed to bile salt-rich micelles (16,17). The 6 mM taurocholate in our elution buffer maintained the integrity of each cholesterol carrier under these gel filtration conditions.

Table 1 compares the separation of vesicles and micelles from three different hamster biles using sucrose density gradient ultracentrifugation and gel filtration column chromatography. We were limited to one gel filtration column as only 50–75 μL of bile was obtained from each hamster. We have previously reported that the percentage cholesterol in vesicles from triplicate separations using gel filtration chromatography were similar [34 ± 4%, mean ± SD] (9). Sample a (Table 1) was not lithogenic (CSI 57%); gel filtration column chromatograph revealed no cholesterol in vesicles while ultracentrifugation showed 8% cholesterol in the vesicular region. Samples b and c (Table 1) were lithogenic (CSI 104 and 114, respectively). Gel filtration and ultracentrifugation showed 42 vs. 44% vesicles for sample b and 50 vs. 46% vesicles for sample c. These results indicate close agreement (better than 90%) between two independent analytical procedures.

In conclusion, we have developed a reliable, reproducible, and rapid ultracentrifugation method for the separation and quantitation of vesicles and micelles in microliter amounts of bile.

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Effect of Degree of Unsaturation in Dietary Fatty Acids on Arachidonic Acid Mobilization by Peritoneal Macrophages

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ABSTRACT: Cells from rats fed with a tripalmitin diet showed a depletion of phospholipid arachidonate and n-3 fatty acids such as eicosapentaenoic and docosahexaenoic acids (EPA and DHA). In rats fed fish oil diet, a significant reduction in arachidonic acid (AA) content was observed whereas EPA and DHA were incorporated into membranes lipids. These changes in lipid composition of membranes did not affect cellular adherence, phagocytic capability, or [^{3}H]AA incorporation. However, both tripalmitin and fish oil diets induced a decrease in [^{3}H]AA mobilization stimulated by 4β-phorbol-12-myristate 13-acetate, A23187, or opsonized-zymosan in rat peritoneal macrophages. These results demonstrate that the antiinflammatory effects of essential fatty acids deficiency or n-3 enrichment diets may be associated with a decreased AA mobilization in resident rat peritoneal macrophages treated with proinflammatory agents.

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Linoleic acid and α-linolenic acid are considered to be essential fatty acids (EFA) for growth and development in humans and animals (1–3). One of the main biochemical functions of the EFA is participated in the control of membrane-associated phenomena such as transport, enzyme activities, and receptor function (4–6). In addition, EFA can affect the production of eicosanoids, which are known to influence cell function. Following dietary deprivation of these fatty acids, a state of EFA deficiency is induced, which is characterized by depressed inflammatory response (7,8).

On the other hand, dietary supplementation with polyunsaturated fatty acids (PUFA) prominent in marine fish oils, which are rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), may participate in many enzymatic steps, thought at one time to be selective for arachidonic acid (AA) (9,10). These fatty acids have been reported to attenuate some of the physiopathological effects of arachidonate metabolites in certain diseases by competing with AA for the enzymes involved in eicosanoid formation. Based on these results, several authors have proposed that dietary fish oil supplementation has potential antiinflammatory effects (11,12). Furthermore, epidemiological studies have demonstrated a lower incidence of psoriasis in Greenland Eskimos than in European controls (13); this has been attributed to the Eskimo's fish diet, which is high in n-3 fatty acids (14). EPA has been demonstrated to be metabolized by both the cyclooxygenase and lipoxygenase pathways by mononuclear cells, producing functionally attenuated analogs of the AA metabolites such as thromboxane A_3 or leukotrienes of the 5 series (15). The biosynthesis of AA metabolites and presumably EPA and DHA metabolites were thought to be initiated in the mononuclear cells by the activation of phospholipase A_2 (PLA_2, E.C. 3.1.1.4.) during cell stimulation (16,17). Thus, PLA_2 is the main rate-limiting enzyme responsible for the mobilization of AA. However, we have to consider that sequential action of a phospholipase C and diacylglycerol lipase (18) or phospholipase D, phosphatidic acid phosphatase, and a diacylglycerol lipase (19) could also induce AA release.

Our purpose was to determine the extent of AA mobilization in resident peritoneal macrophages from rats fed with diets that differed in fatty acid composition.

MATERIALS AND METHODS

Materials. [^{3}H]AA (180–240 Ci/mmol) was obtained from DuPont-NEN (Boston, MA). 4β-Phorbol-12-myristate 13-acetate (PMA), calcium ionophore A23187, and zymosan A were purchased from Sigma Chemical Co. (St. Louis, MO). Opsonized-zymosan was prepared according to Bonney *et al.* (20). All other reagents were of analytical grade.

Animals and diets. Twelve male weanling Sprague-Dawley rats were divided into three homogeneous groups after weaning. Food and tap water were provided *ad libitum*, and the rats were weighed weekly. For 16 wk they were fed isoenergetic semisynthetic diets (INRA, Jouy-en-Josas, France). The nutrient composition of diets was casein (22%), DL-methionine (0.1%), mineral mix (4.5%), vitamin mix (1%), cellulose 2%, corn starch 43.6%, sucrose 21.8%, and 5% of

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Abbreviations: AA, arachidonic acid (20:4n-6); DHA, docosahexaenoic acid (22:6n-3); EFA, essential fatty acids; EPA, eicosapentaenoic acid (20:5n-3); PLA_2, phospholipase A_2; PMA, 4β-phorbol-12-myristate 13-acetate; PUFA, polyunsaturated fatty acid.

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lipids of either standard (2.5% rapeseed oil and 2.5% peanut oil), tripalmitin or fish oil (sardine oil; Afampes 160 from Afamsa, Vigo, Spain). Thus, the three diets differed in their fatty acid composition (Table 1). All diets contained 100 IU/kg of vitamin E, 50 IU/kg were carried-over from oils in order to avoid the PUFA peroxidation, and 50 IU/kg were conventionally added. Diets were stored under vacuum, wrapped in opaque polyethylene foil, stored at 4°C, and were given to the animals daily. Uneaten food remains were removed from the cage each day. Animal care standards and experimental protocols were in accordance with the E.C. guidelines.

Macrophages. Resident peritoneal macrophages were collected from rats killed by carbon dioxide asphyxiation, and the peritoneal cavity was lavaged as described previously (21). Differential cell counts of macrophages were performed microscopically using May Grünwald-Giemsa solution, and $20 \pm 2 \times 10^6$ resident peritoneal macrophages were recovered per rat. Cell viability was measured by the Trypan blue exclusion test. Macrophages were further purified by adherence to plastic tissue culture plates (21). Previous experiments demonstrated that [^{3}H]AA mobilization was triggered simply by contact between cell and plastic substrate (22). Besides, cell activation triggered by cellular adherence disappeared within 16–20 h. In consideration of these results, functional and biochemical assays with [^{3}H]AA labelled macrophages were performed 24 h after cells were purified by adherence to plastic surface.

Peritoneal macrophages adherence. To determine the cellular adherence, macrophages from peritoneal cavity lavaged were counted microscopically. Then, macrophages (10^6 cells/well) were allowed to adhere for a 1-h period in plastic 12-well cluster dishes (Costar, Cambridge, MA), after which the supernatant was removed and nonadhered mononuclear cells were determined using May Grünwald's solution. Cellular adherence was expressed as the percentage of macrophages adhered to plastic.

Phagocytic index. Phagocytic indices were determined by phase contrast microscopy of cells fixed in 2.5% (wt/vol) glutaraldehyde after the ingestion of zymosan (23). Phagosome–lysosome fusion was evaluated by fluorescence microscopy using acridine orange by the method of Hart and Young (24). Then 10^6 macrophages per well were cultured for 24 h in 12-well cluster dishes in RPMI 1640 containing 10% fetal calf serum and antibiotics. Then, cells were pulsed for 20 min with acridine orange, which is concentrated in lysosomes, causing them to appear orange by fluorescence. After zymosan ingestion, zymosan particles are first seen as green inclusions within the cell. Upon fusion of the phagosome with lysosomes, the zymosan becomes stained with acridine orange, resulting in its changing to an orange fluorescence. The phagocytic index was the ratio of orange fluorescing vs. nonfluorescing cells.

TABLE 1
Fatty Acid Composition of Experimental Diets

Fatty acid	Standard	Tripalmitin	Fish oil
14:0	0	0	0.476
16:0	0.375	5	0.950
16:1n-7	0	0	0.547
18:0	0.125	0	0.167
18:1n-9	2.925	0	0.780
18:2n-6	1.075	0	0.176
18:3n-3	0.225	0	0
20:0	0.075	0	0
20:1n-9	0	0	0.144
20:5n-3	0	0	0.931
22:0	0.075	0	0
22:1n-9	0.050	0	0.065
22:5n-3	0	0	0.107
22:6n-3	0	0	0.315

Fatty acid analysis. Cells from peritoneal lavage were adhered to cluster wells (5×10^6 cells/well) for 1 h, in RPMI 1640 (Gibco, Grand Island, NY). Macrophages were removed by treatment with trypsin/EDTA, and lipids were extracted sequentially (25) with chloroform/methanol (1:2, vol/vol), chloroform/saline (1:1, vol/vol), and one part chloroform (2 ×) (25). Total phospholipids were separated by thin-layer chromatography silica gel plates DC-Alufolien (Merck, Darmstadt, Germany) using the solvent system dietyl ether/acetone (90:30, vol/vol). The fatty acid composition of total phospholipids was transmethylated (26) and was determined by gas chromatography following the procedure described by García *et al.* (27). Briefly, fatty acid methyl esters dissolved in hexane were analyzed using a Packard Model 427 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame-ionization detector and a CP WAX 52 CB bonded fused silica capillary column (Chromopack, Les Ulis, France) (50 m × 0.2 mm internal diameter). Hydrogen was the carrier gas, with a flow rate of 1–2 mL/min. The detector and the injector were, respectively, set at 300 and 260°C, and the oven temperature was programmed from 140–225°C at a heating rate of 6°C/min. Peaks were identified by comparison of retention times obtained from equivalent methylated standard mixture injections (Sigma Chemical Co.), and concentrations were calculated from the peak areas obtained by a Delsi (ENICA 10) integrator (Delasi Instruments, Suresnes, France). Results are expressed as percentages of the total fatty acid content.

Incorporation and release of AA. After a 20-h incubation of macrophages (10^6 cells/well) in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Gibco) and antibiotics (100 units of penicillin/mL and 100 μg/mL of streptomycin), the medium was removed and replaced with 0.5 mL of RPMI 1640 containing 0.1 μCi of [^{3}H]AA. Samples were incubated for another 6 h at 37°C. Cells were then washed three times in medium containing 0.5% bovine serum albumin to remove unincorporated [^{3}H]AA. To determine the [^{3}H]AA incorporated into the lipid fraction, cellular lipids were extracted by the method of Bligh and Dyer (28), and radioactivity was measured. The labelled cells were incubated with stimulating agents for 2 h. Then, the medium was removed for analysis of radioactivity released. At the end of

each experiment, the cell monolayer was overlaid with 0.1% Triton-X100, and the cells were scraped off the dishes. Finally, radioactivity present in medium and cell fraction was measured by scintillation counting, using a Packard Tri-Carb 1500 counter.

Statistical analysis. All experimental determinations were performed at least three times to ensure consistency of the observations. Results were expressed as mean ± SEM. Significance of differences between data points and control was determined using the (two-tailed) Student's *t*-test with $P < 0.05$ confidence limits.

RESULTS

We did not observe significant differences in food consumption or growth rates due to diet in any of the three groups of rats. Thus, the total food consumption during the 16-wk period, expressed as kg diet/rat, were 2.6 ± 0.1 for the standard diet group, 3.0 ± 0.2 for the tripalmitin diet group, and 2.5 ± 0.1 for the fish oil diet group. The final body weights were 620 ± 32 g for standard diet animals, 585 ± 34 g for rats fed the tripalmitin diet, and 612 ± 14 g in the fish oil group.

The cells from rats deprived of dietary PUFA by an exclusive tripalmitin diet exhibited a depletion of phospholipid arachidonate (20:4n-6, 50%), EPA (20:5n-3, 36%), and DHA (22:6n-3, 50%), although the AA/EPA ratio was approximately the same (Table 2). Furthermore, an accumulation of monounsaturated fatty acid was also observed (Table 2), in agreement with the results described by Lefkowith *et al.* (29).

In fish oil-fed rats, EPA and DHA were incorporated into membrane lipids and their fatty acid composition revealed several significant changes (Table 2). Thus, the fish oil diet induced a significant reduction in AA content (57%) and concomitant 34- and 1.7-fold rises in EPA and DHA content vs. rats fed with standard diet. Moreover, the AA/EPA ratio was clearly reduced in fish oil-fed rats.

Cell viability was greater than 90% in the three experimental groups and was not modified by dietary manipulation. The percentage of macrophage adherence on plastic culture dishes was similar in the three diet groups (Fig. 1). Similarly, there were no significant differences in a [^{3}H]AA incorporation into lipids of macrophages between diet groups (Fig. 1). Thus, the macrophages incorporated approximately 38% of the [^{3}H]AA under our experimental conditions. Similarly, the changes in lipid composition of membranes by diet did not modify phagocytic capability of the cells (Fig. 1).

The release of [^{3}H]AA from cellular phospholipids after macrophage activation was an early, essential response to stimuli. Thus, PMA (10 μM), ionophore A23187 (1 μM), and opsonized-zymosan (150 μg/mL) were able to induce a significant [^{3}H]AA mobilization (32.5 ± 0.7%, 29.2 ± 2.2%, and 22.3 ± 1.8%, respectively) in prelabelled macrophages obtained from rats fed with standard diet. Similarly, under these same activation conditions, when we used macrophages obtained from rats fed with tripalmitin diet or fish oil diet, the levels of released [^{3}H]AA were inhibited, as compared with cells from rats fed standard diet. This effect was significant when cells were stimulated with PMA or ionophore A23187 in the tripalmitin group and when macrophages were activated with PMA, calcium ionophore A23187, or opsonized-zymosan in the fish oil group (Fig. 2).

DISCUSSION

The phospholipids of macrophages are highly enriched in AA, and under *in vitro* conditions these cells can release AA resulting in extracellular concentrations of AA metabolites (30). The release and metabolism of AA from cellular phospholipids after cell activation are early, essential responses to initiate the inflammatory processes. Since the macrophage is one of the main cellular constituents of the inflammatory lesion, the effect of a PUFA-deprived diet and a fish oil supplementation diet on AA mobilization was examined in resident rat peritoneal macrophages.

TABLE 2
Fatty Acid Composition of Total Phospholipids (% of total fatty acids) in Cellular Membranes

	Diets		
Fatty acid[a]	Standard	Tripalmitin	Fish oil
18:2n-6	5.93 ± 0.49	2.15 ± 0.12[b]	3.34 ± 0.27[b]
18:3n-3	0	0	0.01 ± 0.01[b]
20:3n-9	0.54 ± 0.04	11.53 ± 0.42[b]	0.68 ± 0.05
20:4n-6	28.05 ± 0.53	13.91 ± 0.58[b]	12.62 ± 0.45[b]
20:5n-3	0.22 ± 0.06	0.14 ± 0.02	7.57 ± 0.64[b]
22:6n-3	6.53 ± 0.38	3.27 ± 0.10[b]	11.44 ± 0.43[b]
Total SFA	46.31 ± 0.24	45.70 ± 0.68	47.63 ± 0.59
Total MUFA	6.56 ± 0.47	13.75 ± 0.84[b]	11.83 ± 0.49[b]
Total PUFA	47.13 ± 0.85	40.55 ± 0.64[b]	40.54 ± 0.73[b]
20:4n-6/20:5n-3	127.34 ± 19.56	102.20 ± 18.42	1.60 ± 0.19[b]
Triene/tetraene	0.04 ± 0.01	09.88 ± 0.03[b]	0.11 ± 0.01[b]

[a]Cell lipids were extracted as described in the Materials and Methods section by the Folch method. The fatty acid composition of phospholipids was analyzed as fatty acid methyl esters by gas chromatography. Each value, expressed as percentage with respect to total fatty acid of total phospholipids, is the mean ± SEM of duplicate determinations from four rats; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
[b]Significant difference from the standard diet group ($P < 0.05$).

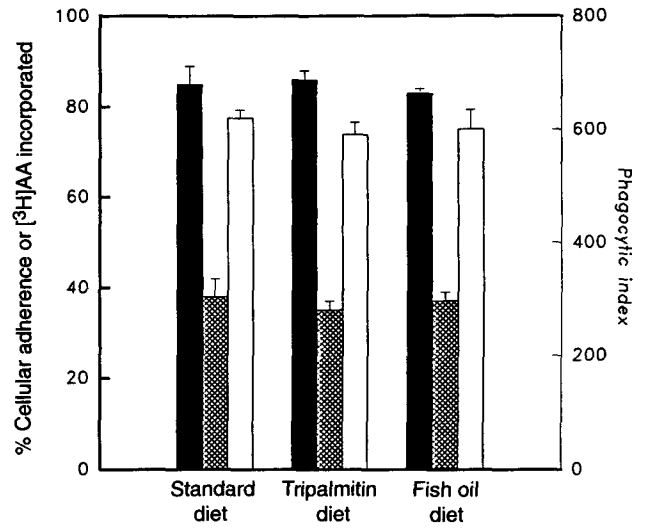


FIG. 1. Effect of tripalmitin or fish oil diets on cellular adherence (closed box), [^{3}H] arachidonic acid (AA) incorporation (cross-hatched box), and phagocytic capability (open box) in rat resident peritoneal macrophages. The data are the means ± SEM of four animals.

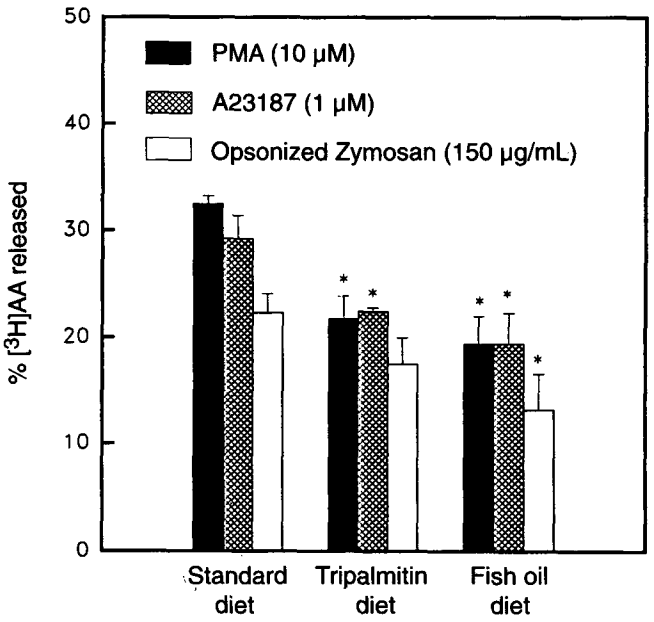


FIG. 2. Effect of tripalmitin or fish oil diet on [^{3}H] arachidonic acid (AA) mobilization induced by 4β-phorbol-12-myristate 13-acetate (PMA) (10 μM), A23187 (1 μM) or opsonized-zymosan (150 μg/mL). Cells labelled with [^{3}H]AA were incubated with agonist for 2 h. The spontaneous [^{3}H]AA released by nonstimulated cells was 5.6 ± 1.2%. The data are the means ± SEM of four animals. *$P < 0.05$, vs. standard diet group.

The fatty acid composition of cell membranes is determined by the fatty acid content of the diet (31). Because synthesis of certain inflammatory mediators may be reduced when membrane fatty acid content is altered, numerous studies have been performed to identify the effects of specific dietary manipulations. Exclusive provision of tripalmitin in the diet induces a deficiency in EFA as indicated by the triene/tetraene index (32). In EFA deficiency diet group, the levels of α-linolenic and linoleic acids are reduced to a point that 18:1 competes for Δ6 desaturase resulting in generation of 20:3n-9 that replaces longer chain n-3 and n-6 fatty acid derivatives. In contrast to EFA deficiency, provision of a fish oil diet induced significant changes in fatty acid composition of membrane phospholipids under our experimental conditions. These changes followed the same trends demonstrated in other phagocytic cells such as neutrophils (33,34).

Furthermore, we found that both tripalmitin and fish oil diets induced a decrease in [^{3}H]AA mobilization stimulated by soluble agents (PMA, A23187) or by a phagocytic stimulus such as zymosan. However, the changes induced by diets in lipid composition of membranes did not disturb other cell functions such as adherence or phagocytic capability.

The inability to alter membrane fluidity in response to changes in membrane fatty acid composition in neutrophils and monocytes (33) could indicate that the modulation of AA release by dietary modifications may be attributable to factors other than alteration of the physical properties of cell membranes.

It is important to note that tripalmitin and fish oil diets induced a significant decrease in cell membrane AA, although the cells of these animals incorporated [^{3}H]AA to the same extent as cells of standard diet-fed animals. However, macrophages isolated from rats fed fish oil suffered a more significant reduction of their capacity to release [^{3}H]AA. These results could indicate that AA mobilization is, at least in part, regulated by the fatty acid composition of the phospholipid bilayer, but perhaps not by substrate availability. Thus, Ballou and Cheung (35) have demonstrated *in vitro* that micromolar concentrations of some PUFA such as DHA can inhibit the activity of semipurified PLA_2. Additionally, increase in the content of some PUFA in microsomal membrane preparations of white blood cell (36) and liver (37) decreased PLA_2 activity. This action was also suggested in a study of the generation of platelet-activating factor by monocytes from volunteers who were fed a fish oil supplement (33).

The efficacy of the dietary manipulations on inflammation (11,12,38,39) is compatible with the view that diet supplemented with EPA- and DHA-enriched oils can exert profound effects on lipid fatty acid compositions, as well as on the endogenous release of AA and biosynthesis of the pro-inflammatory eicosanoids. Synthesis of pro-inflammatory arachidonate metabolites can be affected not only by EPA or DHA competition for the cyclooxygenase and lipoxygenase pathways, but also by the availability of substrate, AA. We hypothesize that the observed anti-inflammatory effect of specific fatty acid manipulations may be associated with a decreased AA mobilization as a consequence of impairment of PLA_2 activity induced by the changes in fatty acid composition of cell membranes.

ACKNOWLEDGMENTS

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ANNOUNCEMENTS

SPECIAL *LIPIDS* SUPPLEMENT—PROCEEDINGS OF THE ISSFAL 2ND INTERNATIONAL CONGRESS

Extra copies of the March *Lipids* Supplement—"Fatty Acids and Lipids from Cell Biology to Human Disease," the Proceedings of the 2nd International Congress of the ISSFAL (International Society for the Study of Fatty Acids and Lipids)—which includes 57 full manuscripts from participants in oral presentations at the Congress (332 total pages), are available for individual purchase at $60 each. Information: AOCS Press, P.O. Box 3489, Champaign, IL 61826-3489 (Phone: 1-217-359-2344, extension 128; Fax: 1-217-351-8091).

CALENDAR

July 1996

European Thrombosis Research Organisation and the International Society for the Study of Fatty Acids and Lipids in cooperation with Summer University of Maastricht, July 10–12, 1996, University of Maastricht, The Netherlands, presents "Lipids, Membranes and Thrombosis (fundamental basis of cardiovascular disease and its prevention)." Information: Gerard Hornstra, Department of Human Biology, P.O. Box 616, 6200MD Maastricht, The Netherlands (Phone: +31-43-88 16 25; Fax: +31-43-67 09 76; E-mail: G. Hornstra @ HB.RuLimburg.NL).

September 1996

37th International Conference on the Biochemistry of Lipids, Antwerp, Belgium, Sept. 4–7, 1996. Themes: enzymes and transfer proteins related to lipoprotein metabolism, aspects of protein lipidation, and peroxisomal lipid metabolism. Poster communications may relate to the three conference themes or any other area of lipid metabolism. Information: Dr. P. van Dyck, Secretariat 37th ICBL, Department of Biochemistry, Physiology and Genetics, Division of Human Biochemistry, State University Centre Antwerp, Groenenborgerlaan 171, B2020 Antwerp, Belgium (Phone: 32 3 218 0329; Fax: 32 3 218 0326).

7th International Congress on Phospholipids, Sept. 9–11, 1996, Brussels, Belgium. Program topic areas include Growth, Death and Cancer; Choline, Choline Phospholipids and the Brain; Importance of Phospholipids in Health and Disease. Information: AOCS Meetings Department, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

IV. International Dermatology Symposion Berlin, Sebaceous Gland and Its Disorders, Basic and Clinical Research, Clinical Entities and Treatment, Sept. 13–15, 1996, University Medical Center Benjamin Franklin. Information: Docent Dr. Ch. C. Zouboulis, Department of Dermatology, University Medical Center Benjamin Franklin, The Free University of Berlin, Hindenburgdamm 20, D-12200 Berlin, Germany (Phone: 49-30-8445-2808; Fax: 49-30-8445-4262).

Training in Free Radical Methodologies, Sept. 23–27, 1996, CERLIB, University of Grenoble, Grenoble, France. Production, damages, repair: ESR, nitric oxide, DNA oxidation, DNA repair, apoptosis, chemical-trapping, lipoprotein oxidation, protein oxidation, cellular production and toxicity. Information: Arlette Alcaraz, Laboratoire de Biochimie C, CHU Grenoble BP 217, 38043 Grenoble Cédex 9, France (Phone: 33 76 76 57 54; Fax: 33 76 76 56 64; E-mail: CERLIB@ujf-grenoble.fr).

October 1996

Frontiers in Lipid and Lipoprotein Research: Basic Science, Analytical, Clinical, and Public Health Applications, Oct. 4–6, 1996, Stouffer Renaissance Hotel, Dallas, Texas. Sponsored by the Lipids and Lipoproteins Division of the American Association for Clinical Chemistry, the Centers for Disease Control and Prevention, and the American Heart Association. An integrated review of relevant recent advances in basic science, measurement technology, epidemiological and clinical research, and public health issues will be presented. This review will help define state-of-the-art techniques for understanding and preventing morbidity and mortality due to heart disease. Information: Paula M. Steiner, Medical Research Laboratories, 2 Tesseneer Dr., Highland Heights, KY 41076 (Phone: 606-781-8877, Extension 252; Fax: 606-781-9310; E-mail: lipf@cehehll.em.cdc.gov).

American College of Nutrition, 37th Annual Meeting, Oct. 11–13, 1996, San Francisco, California (Phone: 212-777-1037; Fax: 212-777-1103).

Certification Board for Nutrition Specialists, Oct. 11, 1996, San Francisco, California. Certifying examination for nutritionists and dieticians with advanced degrees. Information: CBNS, 1562 First Avenue, Box #258, New York, NY 10028 (Phone: 212-332-0774 (leave message); Fax: 212-777-1103).

Seminar: Interconversion of Polyunsaturated Fatty Acids, and Their Functions in Membranes, Oslo, Norway, Oct. 11–12, 1996. Information: Georg Lambertsen, Secretary, Lipidforum, Breistølen 1, N-5035, Bergen, Norway (Phone: 4755 + 32 89 56; Fax: 47 55 + 32 57 24).

November 1996

AOCS Conference on PUFA in Infant Nutrition: Consensus and Controversies, Nov. 7–9, 1996, Barcelona, Spain. Information: Susan E. Carlson (E-mail: scarlson@utmem1.utmem.edu); Robert A. Gibson (E-mail: rgibson@paed_nnu.fmc.flinders.edu.au); Berthold Koletzko (Fax: 49-89-5160-4733); or AOCS Meetings Department, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

March 1997

CERLIB 3rd Winter Research Conferences, Les Arcs 1800, France, March 9–14, 1997. Proteins and Free Radicals, from Radical Enzymes to Damages; Oxidative Effects of Radiations on Biomolecules and Cells; Oxidative Stress and Apoptosis. Information: Arlette Alcaraz, Laboratoire de Biochimie C, CHU Grenoble BP 217, 38043 Grenoble Cédex 9, France (Phone: 33 76 76 57 54; Fax: 33 76 76 56 64; E-mail: CERLIB@ujf-grenoble.fr).

June 1997

The 19th Nordic Lipid Symposium, Ronneby, Sweden, June 15–18, 1997. Information: Georg Lambertsen, Secretary, Lipidforum, Breistølen 1, N-5035, Bergen, Norway (Phone: 47 55 + 32 89 56; Fax: 47 55 + 32 57 24).

July–August 1997

Fourth International Congress on Essential Fatty Acids and Eicosanoids, July 27–August 1, 1997, Edinburgh, Scotland. Information: Secretariat, c/o Meeting Makers, 50 George St., Glasgow G1 1QE, Scotland, United Kingdom.

Hydroperoxides of Erythrocyte Phospholipid Molecular Species Formed by Lipoxygenase Correlate with α-Tocopherol Levels

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ABSTRACT: The hydroperoxides corresponding to the main molecular species of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were determined after lipoxygenase treatment of erythrocyte membranes from healthy children. This work was a preliminary study prior to applying this analytical procedure to erythrocyte membranes from children with diseases associated with vitamin E deficiency. The total molecular species corresponding to 20:4 and 22:6 associated with 16:0 and 18:0 were significantly higher in PE (26.94 ± 4.70 nmol/mg protein) than in PC (20.14 ± 6.70 nmol/mg protein); these concentrations represented 63% of the total molecular species in PE and 22% in PC. However, the concentrations of hydroperoxides produced from these polyunsaturated fatty acid molecular species were in the same order of magnitude in PC (3.98 ± 1.56 nmol/mg protein) and in PE (3.61 ± 1.63 nmol/mg protein). In contrast, the molecular species concentrations containing two double bonds, such as 16:0/18:2 and 18:0/18:2 and their corresponding hydroperoxides, were clearly more elevated in PC than in PE. There was a positive relationship between the concentrations of α-tocopherol and each hydroperoxide of PC and PE, and this association was particularly strong in PE ($P \leq 0.0001$). These results suggest that α-tocopherol exerts a stabilizing effect toward hydroperoxides, limiting their further degradation into peroxyl radicals. The protective effect of α-tocopherol could be more effective in PE because more polyunsaturated fatty acids were present.

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Abbreviations: GLC, gas–liquid chromatography; GPx, glutathione peroxidase; 15-HPETE, 15-hydroperoxyeicosatetraenoic acid; HPLC, high-performance liquid chromatography; PC, phosphatidylcholine; PE phosphatidylethanolamine; 16:0/18:2 PC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-PC; 16:0/20:4 PC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-PC; 16:0/22:6 PC, 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-PC; 18:0/18:2 PC, 1-stearoyl-2-linoleoyl-*sn*-glycero-3-PC; 18:0/20:4 PC, 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-PC, 18:0/22:6 PC, 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-PC; 16:0/18:2 PE, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-PE; 16:0/20:4 PE, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-PE; 16:0/22:6 PE, 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-PE; 18:0/18:2 PE, 1-stearoyl-2-linoleoyl-*sn*-glycero-3-PE; 18:0/20:4 PE, 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-PE; 18:0/22:6 PE, 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-PE; PUFA, polyunsaturated fatty acids, TBARS, thiobarbituric acid reactive substances; UV, ultraviolet.

Many procedures have been described to test the susceptibility of polyunsaturated fatty acids (PUFA) in biomembranes to oxidation. It has been shown that the lipid peroxidation process is affected by the concentration and the degree of unsaturation of fatty acids in the cell membrane (1). Among the free-radical inducers, soybean lipoxygenase (E.C. 1.13.11.12), which catalyzes the oxidation of unsaturated fatty acids (containing a 1,4-*cis,cis* pentadiene system) to hydroperoxides, is the most efficient physiological inducer. Furthermore, esterified fatty acids in phospholipids are acceptable substrates for soybean lipoxygenase (2).

The aim of this work was to study the main molecular species of human erythrocyte membrane phospholipids and their corresponding hydroperoxides after lipoxygenase induction. We attempted to determine if the hydroperoxides produced varied in relation to saturated or unsaturated fatty acids from the *sn*-1 or *sn*-2 position of phosphatidylethanolamine (PE) and phosphatidylcholine (PC). We also attempted to identify relationships between hydroperoxide concentrations and the levels of two main active antioxidant systems, α-tocopherol and glutathione peroxidase (GPx). To obtain a better and more specific marker of lipid peroxidation than malondialdehyde (3,4), we applied to erythrocyte membranes an original technique previously described in soybean phospholipids (5). This simultaneously determines the main molecular species from PE and PC and their corresponding hydroperoxides. We chose the erythrocyte membrane because it is considered a good model for peroxidation processes containing high concentrations of PUFA and has a very effective antioxidant system (1). This work, carried out in erythrocyte from healthy children, represents a preliminary study prior to investigating erythrocyte membranes from children with diseases associated with vitamin E deficiency in an attempt to determine whether such vitamin E deficiency will induce changes in the production of phospholipid hydroperoxides.

MATERIALS AND METHODS

Chromatographic equipment. Gas–liquid chromatography (GLC) was carried out on a Shimadzu (Kyoto, Japan) instru-

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ment equipped with a 25 m × 0.32 mm, 0.25 μm film thickness glass capillary column containing PEG-20 M (Supelco, Bellefonte, PA) and connected to a Delsi-Nermag (Argenteuil, France) integrator. The temperature was programmed from 155 to 250°C (2°C per min). The high-performance liquid chromatography (HPLC) equipment (Thermo Separation Products, Les Ulis, France) included an automatic injector with a 200 μL sample loop, a 250 × 4.6 mm C18 and a 150 × 4.6 mm C8 Kromasil 5 μm (Touzart et Matignon, Les Ulis, France) analytical columns and an ultraviolet (UV)-visible light detector (Thermo Separation Products). The fluorometer (Spectroflow 980 fluorescence detector; Applied Biosystems, Ramsey, NJ) was equipped with a 5 μL flow cell.

Reagents and chemicals. HPLC-grade solvents and analytical-grade chemicals were from Carlo Erba (Milano, Italy) or from Merck (Darmstadt, Germany). Microperoxidase (MP11), isoluminol (6-amino-2,3-dihydro-1,4-phthalazinedione), soybean lipoxygenase (type IB, E.C. 1.13.11.12), and 15-hydroperoxyeicosatetraenoic acid (15-HPETE) were from Sigma (St. Louis, MO).

Ghost preparation. Blood was drawn into heparinized Vacutainer tubes from 12 healthy children (mean age 7 yr, range 1–15 yr) who had fasted overnight. The present study was approved by the local Ethics Committee. Blood was centrifuged for 10 min at 1,000 × *g*, and the cell pellet was washed three times with isotonic buffer. Erythrocyte ghosts were prepared by lysing packed cells in a sevenfold volume of hypotonic phosphate buffer (5 mM, pH 7) with EDTA (1 mM) according to the method of Burton *et al.* (6). The washing procedure was repeated as many times as necessary to obtain white membranes. Ghosts were stored at −70°C until use.

Peroxidation of erythrocyte membranes. All incubations were performed at a membrane protein concentration of 500 μg/mL. The total reaction volume of the membrane pellets was brought up to 1 mL by the addition of 0.1 M borate buffer pH 9.2 containing 10 mM deoxycholic acid. The presence of deoxycholate and also pH 9.2 were necessary for the optimal activity of the enzyme (2). The reaction was started by addition of lipoxygenase at a final concentration of 50 μg/mL. The mixture was incubated with gentle shaking at 30°C for 45 min. The reaction was stopped by addition of molar citric acid to obtain a final pH of 3.5, and the samples were placed on ice for 5 min. A sample was incubated in the same conditions but without lipoxygenase to determine the molecular species of phospholipids before peroxidation.

Lipid extraction and phospholipid separation. Lipids were extracted with chloroform/methanol (2:1, vol/vol). The mixture was partitioned between the chloroform layer (lower organic phase) and the methanol/water layer (upper phase) by centrifugation at 1,500 × *g* for 5 min. After two extractions, the lower chloroform layers were collected, evaporated under a stream of nitrogen, and then dissolved in methanol. Silica-SepPak columns were used to separate phospholipids into PC and PE, using the extraction technique described by Hamilton and Comai (7). The two fractions corresponding to PC and PE were evaporated to dryness, then dissolved in methanol, and injected into the HPLC system. We have previously shown in soybean phospholipids (5) that there was a strong relationship between the concentration of transformed molecular species after peroxidation and the concentration of the corresponding hydroperoxides. Consequently, the chloroform/methanol extraction is suitable for quantitative determination of phospholipid molecular species and hydroperoxides.

Molecular species detection of PC and PE and their corresponding hydroperoxides. The procedure described in detail elsewhere with soybean PC and PE (5) was applied with slight modifications. Separation was done on the liquid chromatograph using two serial analytical columns: a C8 (150 × 4.6 mm) and a C18 (250 × 4.6 mm) Kromasil 5 μm, maintained at 40°C. The mobile phase was 6% 10 mM ammonium acetate, pH 5, and 94% methanol (flow rate 1.5 mL/min). The molecular species of PC and PE were detected at 205 nm before and after 45 min of peroxidation. The eluate was then mixed at a flow rate of 1.5 mL/min with the chemiluminescent reagent, prepared as described by Yamamoto *et al.* (8), with slight modifications. Isoluminol (55 mg) was dissolved in a mixture of methanol/0.1 M borate buffer, pH 9.2 (3:7, vol/vol), and 10 mg/L of microperoxidase was added to the mixture. The solution was passed through a fluorometer used as a photon detector with the excitation source turned off.

Molecular species and hydroperoxide identification—expression of the results. Fatty acid molecular species were identified by GLC with comparison to a commercial standard mixture as follows: 16:0/18:2, 16:0/20:4, 16:0/22:6, 18:0/18:2, 18:0/20:4, and 18:0/22:6 in PC and PE. The corresponding hydroperoxides were identified by peroxidation of the standard molecular species (5). In our conditions of chromatography, both hydroperoxides (16:0/20:4 and 16:0/22:6 or 18:0/20:4 and 18:0/22:6) had the same retention time. It was not possible to separate them even if the experimental conditions were changed including the column, mobile phase, and temperature. Each hydroperoxide was quantified on the basis of the relative sensitivity of the chemiluminescence assay for 15-HPETE, using a coefficient which included the detection limit for the various hydroperoxides relative to 15-HPETE. Under these conditions the correction coefficient was 0.8 for 16:0/20:4 including 16:0/22:6, 0.8 for 18:0/20:4 including 18:0/22:6, and 3.5 for 16:0/18:2 or 18:0/18:2. In erythrocyte membranes, the limit of detection for hydroperoxides containing 16:0/18:2 was 1 nmol/mg protein, and the limit for hydroperoxides containing 16:0/20:4 plus 16:0/22:6 was 0.25 nmol/mg protein. The reproducibility of the hydroperoxide species was 82% ± 8 for PE and 85% ± 7 for PC. Using single molecular species, we verified that the steps of extraction and SepPak separation did not modify the percentage of hydroperoxide recovery (5).

α-Tocopherol and glutathione peroxidase. α-Tocopherol was determined by using an HPLC technique derived from that of Zaspel and Csallany (9). GPx activity was determined according to Paglia and Valentine (10), using Triton X100 to dissolve the membranes. One unit of GPx was defined as 1 μM of substrate converted in 1 min. Malondialdehyde con-

tent was expressed as thiobarbituric acid-reactive substances (TBARS) and was measured by HPLC with fluorescence detection (11).

Statistical analysis. Data were expressed as mean ± SD. The statistical significance of differences was determined by the Mann-Whitney test. Correlation analysis was performed by means of the linear correlation coefficient.

RESULTS

α-Tocopherol, TBARS, and GPx. Before peroxidation the mean concentration (±SD) of α-tocopherol was 483 ± 187 ng/mg protein and showed a large range of values, from 268 to 878 ng/mg protein, which was not related to the children's age. TBARS concentration was 282 ± 52 pmol/mg protein, and that of GPx activity was 35.9 ± 11.6 mU/mg protein. After peroxidation, only TBARS were evaluated, and the mean concentration found was 1680 ± 350 pmol/mg protein. We observed that no α-tocopherol remained after lipoxygenase treatment. There was no relationship between α-tocopherol concentration and either TBARS or GPx activity.

Molecular species. Six main molecular species were separated from PC (Fig. 1A) and PE, and the concentrations of these different molecular species in PE and PC were determined (Table 1). The main molecular species in PC were 16:0/18:2 and 18:0/18:2 which represented 55 and 22%, respectively, of the total molecular species. In PE, 16:0/18:2 corresponded to 29% of the total molecular species and 18:0/18:2 only to 8%. In contrast, the total PUFA 20:4 and 22:6 associated with 16:0 or 18:0 represented 63% of total molecular species in PE and 22% in PC. The concentrations

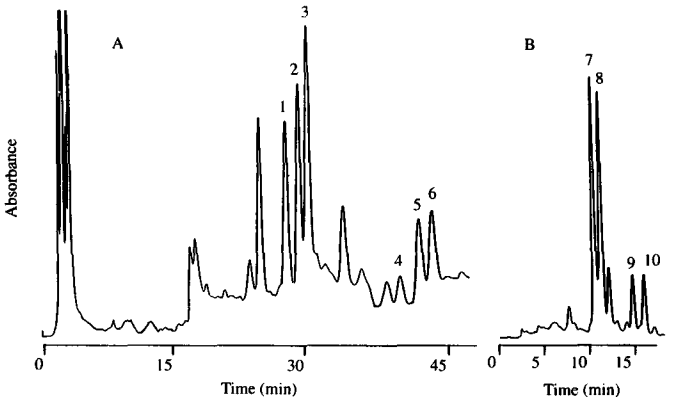


FIG. 1. Separation of phosphatidylcholine (PC) molecular species in human erythrocyte membrane before lipoxygenase treatment (A) and separation of the corresponding hydroperoxides after lipoxygenase treatment (B) using two serial Kromasil C8 and C18 analytical columns (Touzart et Matignon, Les Ulis, France) at 40°C. The mobile phase was methanol/10 mM ammonium acetate (94:6, vol/vol; pH 5.0) delivered isocratically at 1.5 mL/min. Fatty acid molecular species were detected at 205 nm, and hydroperoxides were detected with the chemiluminescence reagent at a flow rate 1.5 mL/min. A, 1 = PC 16:0/22:6, 2 = PC 16:0/20:4, 3 = PC 16:0/18:2, 4 = PC 18:0/22:6, 5 = PC 18:0/20:4, 6 = PC 18:0/18:2. B, 7 = phosphatidylcholine hydroperoxides (PCOOH) 16:0/18:2, 8 = PCOOH 16:0/20:4 + 16:0/22:6, 9 = PCOOH 18:0/18:2, 10 = PCOOH 18:0/20:4 + 18:0/22:6.

of PUFA (20:4 and 22:6) associated with 16:0 were in the same order of magnitude in PC as in PE, but the same PUFA associated with 18:0 was significantly higher in PE than in PC ($P \leq 0.001$). Thus, the total PUFA concentration in PE (26.94 ± 4.70 nmol/mg protein) was significantly higher ($P \leq 0.01$) than in PC (20.14 ± 6.70 nmol/mg protein). After per-

TABLE 1
Molecular Species Concentrations in Phosphatidylcholine (PC) and in Phosphatidylethanolamine (PE) and Their Corresponding Hydroperoxide Concentrations[a] After Lipoxygenase Treatment

	Molecular species before peroxidation (nmol/mg protein)	Percentage molecular species transformed after lipoxygenase	Hydroperoxides after lipoxygenase (nmol/mg protein)	Percentage[b] of hydroperoxides produced after lipoxygenase
PC				
16:0/22:6	3.13 ± 1.23	78	3.98 ± 1.56[c]	36
16:0/20:4	12.87 ± 4.28	67		
16:0/18:2	49.10 ± 9.81	40	9.78 ± 4.13	48
18:0/22:6	0.70 ± 0.37	79	0.84 ± 0.38[d]	26
18:0/20:4	4.23 ± 1.52	63		
18:0/18:2	19.51 ± 6.14	43	2.07 ± 0.64	30
Sum PC	89.03 ± 18.3	47	16.67 ± 6.48	39
PE				
16:0/22:6	3.32 ± 1.08	79	3.61 ± 1.63[c]	30
16:0/20:4	13.38 ± 2.80	69		
16:0/18:2	12.35 ± 3.89	56	1.35 ± 0.64	26
18:0/22:6	1.78 ± 0.57	77	1.71 ± 0.51[d]	26
18:0/20:4	8.46 ± 2.32	60		
18:0/18:2	3.36 ± 1.25	31	Not detected	—
Sum PE	39.30 ± 7.70	67	6.67 ± 2.71	25

[a]Values are mean ± SD from twelve erythrocyte membranes. [b]Percentage of phospholipid hydroperoxides produced was calculated from the concentrations of molecular species transformed after lipoxygenase treatment. [c]Sum of 16:0/22:6 and 16:0/20:4 hydroperoxides. [d]Sum of 18:0/22:6 and 18:0/20:4 hydroperoxides.

oxidation, the amounts of molecular species consumed from PC or PE were in the same order of magnitude for the fatty acid containing more than two double bonds. The biggest difference concerned the 16:0/18:2, which was more transformed in PE (56%) than in PC (40%). However, it is difficult to conclude that PE was more reactive than PC on the basis of this single difference. Therefore, it appears that lipoxygenase is rather nonspecific to molecular species. In the presence of lipoxygenase, the amounts of molecular species transformed increased with the number of double bonds in both PC and PE. For example, in PC, 78% was transformed for 16:0/22:6, 67% for 16:0/20:4, and 40% for 16:0/18:2.

Hydroperoxides. The hydroperoxides corresponding to the main molecular species were detected after lipoxygenase treatment (Fig. 1B), and their concentrations were determined (Table 1). No hydroperoxides were detectable before peroxidation. We have not detected hydroperoxide from the 18:0/18:2 molecular species in PE due to the low concentration of the corresponding molecular species and the low percentage of molecular species transformed.

The amount of hydroperoxides after lipoxygenase induction varied from 0.31 nmol/mg protein to 16 nmol/mg protein according to the molecular species. Also, hydroperoxide concentration showed a wide range of values owing to differences between the different membrane preparations. The total hydroperoxide concentration from PUFA containing more than two double bonds was not significantly different between PC and PE (4.81 ± 1.97 nmol/mg protein and 5.33 ± 2.00 nmol/mg protein, respectively). For the sum of 16:0/20:4 and 16:0/22:6 in PC and in PE, the same molecular species concentration produced in average approximately the same concentrations of hydroperoxides, 3.98 ± 1.56 and 3.61 ± 1.63 nmol/mg protein, respectively. For 18:0/20:4, the hydroperoxide concentration was two times greater in PE than in PC and corresponded to a twofold larger molecular species concentration before peroxidation. In PC, the largest mean hydroperoxide concentration was obtained from 16:0/18:2 (9.78 ± 4.13 nmol/mg protein), and this concentration represented about half of the molecular species transformed. Comparing this last result with that obtained for 18:0/18:2, hydroperoxide concentrations were lower, exhibiting only 30% of the amount of the molecular species transformed. In PE, the mean hydroperoxide concentration produced from 16:0/18:2 was low, 1.35 ± 0.64 nmol/mg protein compared to PC and this concentration represented 26% of the amount of molecular species transformed.

Relationships between antioxidants and hydroperoxides. There was a positive relationship between α-tocopherol before lipoxygenase treatment and each hydroperoxide concentration for PC and PE (Table 2) after lipoxygenase treatment. This relationship is specially marked for the hydroperoxides produced from 16:0/20:4 + 16:0/22:6 molecular species in PE (Fig. 2). In contrast, there was no relationship between the amount of molecular species transformed and α-tocopherol concentrations. Furthermore, there was no link between hydroperoxide concentration and GPx activity in either PC or PE.

TABLE 2
Relationships Between α-Tocopherol Before Lipoxygenase Treatment and Hydroperoxide Concentrations in PE and in PC After Lipoxygenase Treatment

	r^a	P^b
PCOOH-molecular species		
16:0/20:4 + 16:0/22:6	0.844	≤0.0005
16:0/18:2	0.712	≤0.0094
18:0/20:4 + 18:0/22:6	0.629	≤0.028
18:0/18:2	0.709	≤0.01
PEOOH-molecular species		
16:0/20:4 + 16:0/22:6	0.966	≤0.0001
16:0/18:2	0.922	≤0.0001
18:0/20:4 + 18:0/22:6	0.815	≤0.001

[a]Coefficient of correlation. [b]Probability values. [c]PCOOH, PC hydroperoxides; PEOOH, PE hydroperoxides. See Table 1 for other abbreviations.

DISCUSSION

We studied the main molecular species of human erythrocyte membrane phospholipids and their corresponding hydroperoxides after lipoxygenase treatment. Only total evaluation of hydroperoxides from PC and PE was reported in the literature with different methods of detection (8,12,13). The method described in the present work represents a significant improvement over the usual TBARS determination, which only accounts for a small percentage of fatty acids consumed compared to the amount of hydroperoxides recovered (14). The experimental model used in this work, phospholipid–bile salt micelles, does not directly reflect the event in biological membranes, but the antioxidant effect of α-tocopherol was

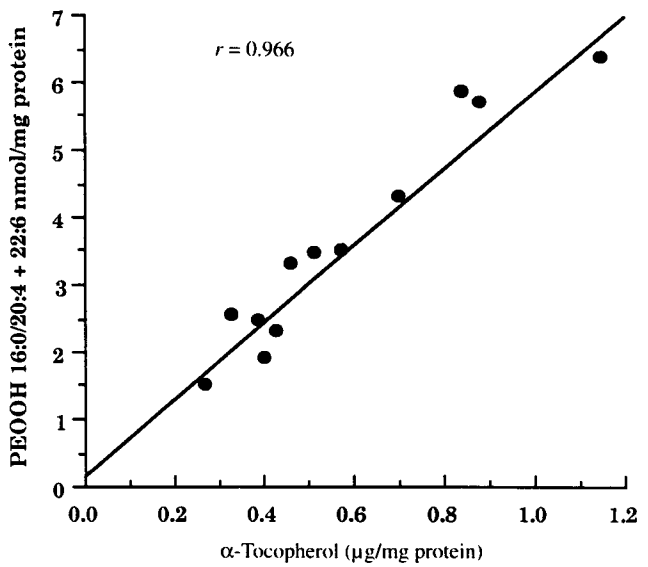


FIG. 2. Relationship between the concentration of α-tocopherol before lipoxygenase treatment and the concentration of phosphatidylethanolamine (PE) 16:0/20:4 + 16:0/22:6 hydroperoxides (PEOOH) after lipoxygenase treatment in human erythrocyte membrane.

observed either in micelles or in biological membrane (15). Therefore, determination of hydroperoxide molecular species from the main classes of phospholipids should improve our knowledge of the peroxidation process in membrane.

As seen in Table 1, the main differences observed between PC and PE concerned the peroxidation of 16:0/18:2. The amount of this molecular species transformed by lipoxygenase was higher in PE (56%) than in PC (40%). However, only 26% was recovered as hydroperoxides in PE compared to 48% in PC. This result is consistent with the recent study of Guo *et al.* (16) using 2,2′-azo*bis*(2-amidinopropane) dihydrochloride as the initiator of the peroxidation process in erythocyte membranes. According to this author, the high susceptibility of 16:0/18:2 to peroxidation in PE might be due to an attack on another part of the phospholipid including oxidation of primary amino groups. These last oxidation products, which represent about 50% of the molecular species transformed (16), are not recovered as hydroperoxides and could not be detected in our experimental conditions.

The amount of molecular species transformed from PC and PE was not related to the concentrations of the molecular species prior to the peroxidation but to the number of double bonds. These results confirm the greatest susceptibility of PUFA to the peroxidation process as a function of fatty acid unsaturation (1). The percentages of the molecular species transformed containing fatty acids with more than two double bonds, and hydroperoxides produced were in the same order of magnitude in PC as in PE. Despite a significantly higher concentration of these PUFA in PE, we observe about the same concentration of hydroperoxides produced. As PUFA are a good target for free radicals, the corresponding hydroperoxides would be expected to be more abundant in PE than PC. The link between unsaturation of fatty acids in membane phospholipids and hydroperoxide production is unclear (17,18). Other factors probably influence the degree of peroxidation, such as the different antioxidant systems located in the membrane, especially α-tocopherol.

The fundamental role of α-tocopherol in the membrane was evident when we considered the relationships between the concentrations of α-tocopherol and hydroperoxides: the higher the concentration of α-tocopherol, the higher the concentration of hydroperoxides. This is surprising given the antioxidant role of this vitamin. However, the role of α-tocopherol in the different steps of lipid peroxidation process could partly explain this strong relationship. Because α-tocopherol acts as a hydrogen donator, it scavenges chain-propagating radicals, suppresses peroxyl radicals, and thus contributes to stabilizing the hydroperoxides produced. In the absence of effective hydrogen donators, such as vitamin E, peroxyl radicals increase and their transformation into hydroperoxides is reduced. Peroxyl radicals from fatty acids with more than two double bonds undergo intramolecular cyclization (19). In the case of peroxyl radicals from linoleic acid whereby intramolecular cyclization is impossible, other reactions occur such as peroxide-linked dimers with hydroperoxides (20) concurring to minimize hydroperoxide production.

Different arguments strengthen the specific effect of α-tocopherol toward hydroperoxides formation. First, GPx activity in the presence of deoxycholate was inhibited during the lipoxygenase treatment (21). Second, the extent of peroxidation corresponding to the amounts of molecular species transformed did not vary with α-tocopherol content.

The inhibitory effect of α-tocopherol toward 15-lipoxygenase *via* a noncompetitive interaction with the enzyme protein (22,23) could also be advanced. Thus, the protective effect of α-tocopherol can occur through the formation of an inactive complex with lipoxygenase, besides the trapping of peroxyl radicals. However, the inhibitory effect was unlikely because our experimental conditions were not suitable (alkaline pH) and the concentration of α-tocopherol found in erythrocyte membranes was not in the inhibitory range (24).

In conclusion, we suggest that α-tocopherol may, *in vivo*, first interact with the lipoxygenase and secondly may stabilize the hydroperoxides, reducing their further decomposition into secondary products in relation with the concentration of α-tocopherol. This protective effect of α-tocopherol should be more efficient in PE where more PUFA were present, as previously suggested (25) for the inner layers of liver mitochondrial membranes. A similar experiment will be carried out using erythrocyte membranes from children with diseases associated with vitamin E deficiency, such as cystic fibrosis and chronic cholestasis. We previously observed vitamin E deficiency in erythrocyte membranes from children with cystic fibrosis who do not have plasma vitamin E deficiency despite vitamin E treatment (Thérond, P., Couturier, M., Lemonnier, F., Demelier, J.F., and Navarro, J., unpublished data). Consequently, it will be interesting to determine whether this vitamin E deficiency induces modification of the peroxidation process involving the main phospholipid classes and molecular species in each. The present method will be used to monitor the effect of vitamin E and fatty acids supplementation on the composition of the membrane phospholipids and to determine what species of hydroperoxides are produced at various vitamin E levels.

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Plasma Concentrations of Dihydro-Vitamin K_1 Following Dietary Intake of a Hydrogenated Vitamin K_1-Rich Vegetable Oil

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ABSTRACT: Dihydro-vitamin K_1 is a dietary form of vitamin K_1 (phylloquinone) produced during the hydrogenation of vegetable oils. To determine if dihydro-vitamin K_1 is present in plasma following dietary intake of a hydrogenated fat, eight healthy adults consumed each of two diets containing 30% of calories from fat, of which 20% was either soybean oil or a partially hydrogenated soybean oil-based stick margarine. Of the fats and oils analyzed, dihydro-vitamin K_1 was only found in the hydrogenated products. The soybean oil diet contained 180 ± 12 μg (mean ± SD) of vitamin K_1/day and nondetectable levels of dihydro-vitamin K_1, whereas the stick margarine diet contained 199 ± 7 μg of vitamin K_1/day and 23 ± 2 μg of dihydro-vitamin K_1/day. After consuming each diet for five weeks, plasma dihydro-vitamin K_1 concentrations were higher ($P = 0.002$) in all eight subjects when consuming the stick margarine diet (0.56 ± 0.33 nmol/L) compared to the soybean oil diet (0.12 ± 0.11 nmol/L). There was no significant change in plasma vitamin K_1 concentrations when the two diets were compared. In conclusion, dihydro-vitamin K_1 is detectable in plasma following dietary intake of a hydrogenated vitamin K_1-rich vegetable oil. *Lipids 31*, 709–713 (1996).

Certain vegetable oils are important dietary sources of vitamin K_1 (phylloquinone), which is the predominant form of vitamin K found in the diet (1,2). Of these vegetable oils, the highest concentrations of vitamin K_1 are measured in soybean, canola, and olive (3). Food consumption data for this century show a general shift from animal to vegetable sources of dietary fat, attributed to health concerns associated with the intake of saturated fats and cholesterol (4). Soybean oil is the most commonly consumed vegetable oil in the American diet, often in a hydrogenated form (5).

We recently described high-performance liquid chromatography (HPLC) methodology for the determination of vitamin K_1 in various food matrices (6). In our analysis of bread, we observed a peak that corresponded to the retention time of 2′,3′-dihydro-vitamin K_1. The identity of dihydro-vitamin K_1 has since been confirmed by gas chromatography/mass spectrometry, along with its conversion from vitamin K_1 during the hydrogenation process (7).

The physiological importance of this observation is dependent on the biological activity of the hydrogenated form of vitamin K. Vitamin K-dependent reactions are related to both the length and the isomeric configuration of the side chain (8). The effect of saturation of the 2′,3′ double bond on the biological activity of vitamin K was examined using the chick bioassay, as reviewed in 1941 by Fieser *et al.* (9). There was an overall loss in potency of the vitamin when saturated, although the data on the magnitude of the reduction in biological activity were inconsistent when compared to the parent compound, vitamin K_1. As this hydrogenated form of vitamin K is abundant in the food supply (10), the biological activity of dihydro-vitamin K_1 in humans needs to be determined. In this paper, we report plasma concentrations of vitamin K_1 and dihydro-vitamin K_1 following dietary intake of known amounts of a hydrogenated and nonhydrogenated vegetable oil.

EXPERIMENTAL PROCEDURES

Study design. This study was a subset of a larger human metabolic study examining the effects of hydrogenated fat intake on lipid profiles. Eight moderately hypercholesteremic, but otherwise healthy participants (three men, five women), were selected for this study. They ranged in ages from 58 to 72 yr (mean = 64 yr). All participants were in good health as determined by a physical examination and screening laboratory results. Exclusion criteria were as follows: (i) use of medications known to affect lipid metabolism or clotting function; (ii) history of renal, hepatic, endocrine, or gastrointestinal disease; and (iii) women currently on postmenopausal estrogen replacement therapy.

All participants consumed each of the two test diets according to a randomized study design. Each diet phase had a duration of five weeks, with a minimum two-week washout period between phases. The diet phases consisted of a mixed diet containing only naturally occurring foods, and were composed of 15% protein, 55% carbohydrate, and 30% of kcals from fat. A rotating three-day menu was used throughout the study. Of the calories derived from fat, 20% was provided by either soybean oil or a commercial soybean oil-based stick

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Abbreviations: FDA, Food and Drug Administration; HPLC, high-performance liquid chromatography; $K_{1(25)}$, 2-methyl-3-(3,7,11,15,19-pentamethyl-2-eicosenyl)-1,4-napthalenedione; RDA, recommended dietary allowance; SPE, solid-phase extraction; TDS, total diet study.

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margarine. The diet was designed to provide nutrient adequacy by meeting the recommended dietary allowance (RDA) for energy, protein, minerals, and vitamins (11). Replicates of each meal (based on a 2,000 kcal intake) were prepared as for consumption, and the entire contents of each single day menu were homogenized in a stainless-steel blender (Waring Products Division, New Hartford, CT). Aliquots were then frozen at −20°C and protected from light until the time of vitamin K_1, dihydro-vitamin K_1, and total fatty acid analyses.

Although study participants were free-living, they were only allowed to eat and drink foods and beverages provided to them by the Metabolic Research Unit at the Jean Mayer USDA Human Nutrition Research Center on Aging (Boston, MA) where all meals were prepared under the supervision of a dietitian. The caloric content of the diet was adjusted to maintain body weight within 1.5 kg throughout the study. Each participant was weighed three times per week. In the fifth week of each diet phase, a single fasting blood sample was drawn, aliquoted, and stored at −70°C until analyses.

The study protocol was approved by the Human Investigation Review Committee of Tufts University, and a written consent form was obtained from each study participant.

Analytical procedures. The solvents used in sample extraction and chromatography were HPLC grade (Fisher Scientific Inc., Springfield, NJ). Vitamin K_1 was purchased from Sigma Chemical Co. (St. Louis, MO). 2′,3′-Dihydro-vitamin K_1 was synthesized by hydrogenation of vitamin K_1 using a modification of the procedure reported by Langenberg and Tjaden (12). The internal standard $K_{1(25)}$ [2-methyl-3-(3,7,11,15,19-pentamethyl-2-eicosenyl)-1,4-napthalenedione] was a gift from Hoffman-La Roche & Co. (Basel, Switzerland). Standard solutions were diluted to known concentrations in 100% methanol and characterized spectrophotometrically and chromatographically. Working solutions were stored at 4°C and protected from light. All procedures involving sample processing and preparation were performed under yellow lighting since vitamin K compounds are sensitive to photodegradation.

Vitamin K_1 and dihydro-vitamin K_1 concentrations in plasma were determined by reverse-phase HPLC using post-column reduction and fluorometric detection as described in detail elsewhere (6,13).

Sample extracts were prepared for injection on the HPLC as follows: 2 mL of plasma were transferred to a disposable borosilicate screw-cap culture tube (16 × 100 mm) to which 4.0 ng (0.02 mL) of the internal standard $K_{1(25)}$ was directly added. Plasma proteins were denatured by adding 4 mL of ethanol and vortexing for 10 s, followed by the addition of 1 mL of H_2O and 12 mL of hexane. Following mixing for 1 min and centrifugation at 1,000 × *g* for 5 min, the upper (hexane) layer was transferred to a disposable borosilicate culture tube (16 × 100 mm) and evaporated to dryness in a centrifugal evaporator (Savant Instruments Inc., Farmingdale, NY). The residue was redissolved in 1 mL of hexane and further processed by two-stage solid-phase extraction (SPE). SPE silica gel columns (3 mL) (J.T. Baker Inc., Phillipsburg, NJ) were preconditioned by a wash of 8 mL of hexane/diethyl ether (93:3, vol/vol), followed by 8 mL of 100% hexane. The 1 mL extract was applied directly onto the preconditioned column packing, followed by a wash with 8 mL of 100% hexane. The phylloquinone-containing fraction was eluted from the SPE columns with 8 mL hexane/diethyl ether (93:3, vol/vol). The eluants were collected into clean 16 × 100 culture tubes and evaporated to dryness. To further purify the lipid extract for injection on the HPLC, a reverse-phase C18 SPE extraction was used. After evaporation, the residue from the eluate of the silica gel SPE columns was dissolved in 200 µL of isopropanol with heat (45°C) for 10 min. The reverse-phase C18 columns (3 mL) (J.T. Baker Inc.) were preconditioned by successive washes with 3 mL of methanol/methylene chloride (80:20, vol/vol), followed by 3 mL of 100% methanol and 3 mL of 100% H_2O. The reconstituted extracts were applied directly to the preconditioned packing. The column was washed with 3 mL of methanol/H_2O (95:5, vol/vol) followed by 3 mL of 100% acetonitrile, and the vitamin K-containing fraction was eluted from the column with 6 mL of methanol/methylene chloride (80:20, vol/vol). The eluant was evaporated to dryness and reconstituted in 25 µL of 100% methylene chloride with swirling to completely dissolve the residue. Immediately after dissolving, 100 µL of methanol (containing 10 mM $ZnCl_2$, 5 mM acetic acid, and 5 mM sodium acetate) was added. A volume of 100 µL was injected for analysis by HPLC.

Every fourth injection was of a calibration standard containing vitamin K_1, dihydro-vitamin K_1, and the internal standard, vitamin $K_{1(25)}$. In addition, a plasma control was analyzed with every sample batch. The coefficients of variation for ten replicates of the control plasma analyzed on the same day were 5.3 and 7.1% for vitamin K_1 and dihydro-vitamin K_1, respectively.

Analytical procedures used for the analyses of metabolic diets, and the individual fats and oils incorporated into diets, have been described elsewhere (6,7). Different hydrogenated fat sources were analyzed for their vitamin K_1 and dihydro-vitamin K_1 concentrations as part of the protocol for the larger human metabolic study. The soybean oil and margarines used in the diets were obtained from a local commercial vendor, with each sample representing a single lot. The *trans* fatty acid content of the diets were analyzed by Best Foods Research and Engineering Center (Union, NJ) (14).

Statistical analysis. A paired samples *t*-test was used to compare differences between the two test diets for both plasma vitamin K_1 and dihydro-vitamin K_1 concentrations. A *P*-value <0.05 was defined as statistically different.

RESULTS

The vitamin K_1 and dihydro-vitamin K_1 concentrations of soybean oil, a variety of hydrogenated fats, and butter are presented in Table 1. Dihydro-vitamin K_1 was only present at detectable levels in the three hydrogenated products, which was supported by corresponding increases in the percentage of total *trans* fatty acid content.

TABLE 1
Vitamin K_1 and Dihydro-Vitamin K_1 Concentrations of Different Oils and Hydrogenated Fats, with Corresponding Total *Trans* Fatty Acid Composition

Description	Vitamin K_1 (μg/100 g ± SD)	Dihydro-vitamin K_1 (μg/100 g ± SD)	Total *trans* fatty acid (%)
Butter	5.4 ± 1.3	nd[a]	2.6
Soybean oil	238 ± 17	nd	0.3
Liquid (soybean-based) margarine	25 ± 1.3	nd	0.6
Tub (soybean-based) margarine	64 ± 0.7	34 ± 1.1	9.4
Commercial partially-hydrogenated cooking fat	212 ± 5.9	96 ± 2.8	13.6
Stick (soybean-based) margarine	50 ± 1.9	132 ± 4.9	26.1

[a]nd = Below the lower limit of detection of 0.01 μg of dihydro-vitamin K_1/100 g.

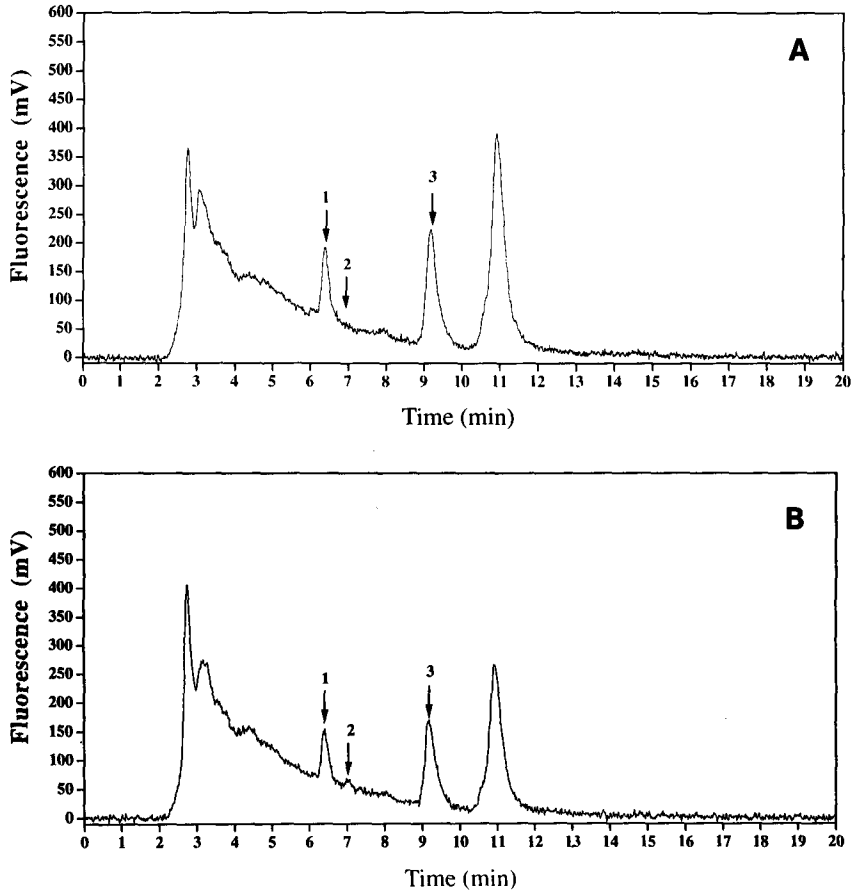


FIG. 1. Representative high-performance liquid chromatography chromatograms of vitamin K_1 and dihydro-vitamin K_1 in human plasma following consumption of (A) soybean oil diet, and (B) stick margarine diet. Peaks correspond to (1) vitamin K_1, (2) dihydro-vitamin K_1, and (3) the internal standard, $K_{1(25)}$.

The mean ± SD daily intake of vitamin K_1 during the soybean oil phase was 180 ± 12 μg, compared to a mean ± SD daily intake of vitamin K_1 during the stick margarine phase of 199 ± 7 μg ($P = 0.08$). In adults, the current daily RDA for vitamin K is 65–80 μg of phylloquinone (11). The stick margarine diet contained a mean ± SD dihydro-vitamin K_1 concentration of 23 ± 2 μg/day, whereas dihydro-vitamin K_1 was not detected in the soybean oil diet. The lower limit of detection was 0.01 μg of dihydro-vitamin K_1/100 g of food. Vegetables, such as lettuce, broccoli, and peas, were the primary dietary sources of vitamin K_1 in the two diets.

Representative chromatograms of plasma vitamin K_1 and dihydro-vitamin K_1 following five weeks of consumption of the soybean oil diet (A) and the stick margarine diet (B) are presented in Figure 1. The dihydro-vitamin K_1 concentrations were significantly higher in all eight study participants in the stick margarine phase than the soybean oil phase ($P = 0.002$) (Fig. 2A). There was no significant difference in plasma vita-

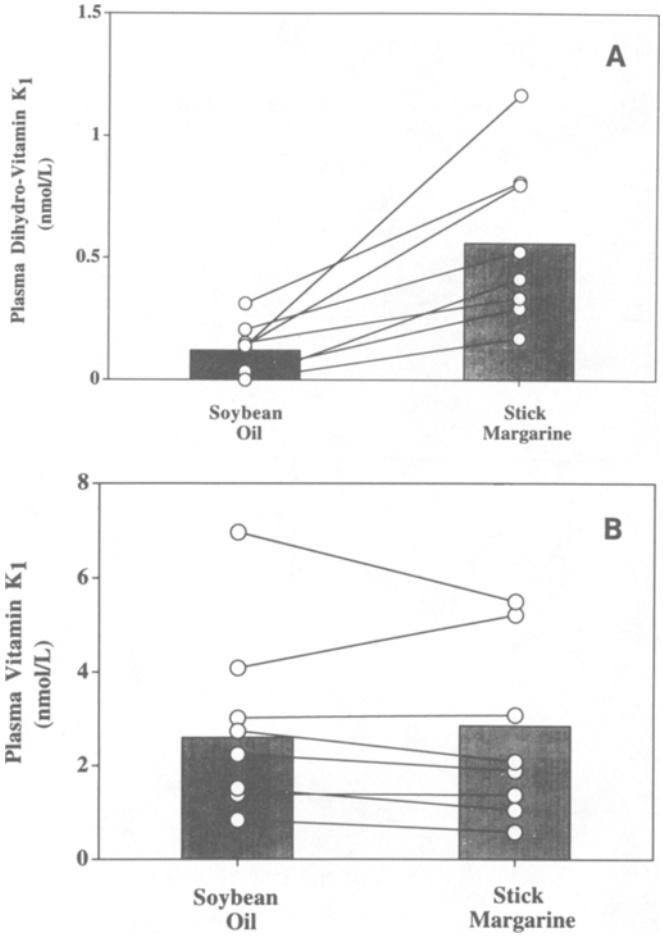


FIG. 2. Comparisons of (A) plasma dihydro-vitamin K_1 concentrations and (B) plasma vitamin K_1 concentrations in each individual in response to a five-week soybean oil diet and a five-week stick margarine diet. The lines connect the paired values for each individual. The bars represent the group mean for each diet: (A) the mean ± SD dihydro-vitamin K_1 concentrations were 0.12 ± 0.11 and 0.56 ± 0.33 nmol/L for the soybean oil and stick margarine diets, respectively ($P = 0.002$), and (B) the mean ± SD vitamin K_1 concentrations were 2.85 ± 1.95 and 2.60 ± 1.86 nmol/L for the soybean oil and stick margarine diets, respectively ($P = 0.41$).

min K_1 concentrations when the two-diet phases were compared ($P = 0.408$) (Fig. 2B).

DISCUSSION

The results of this study show that 2′,3′-dihydro-vitamin K_1 does appear in plasma following the dietary intake of a hydrogenated vitamin K_1-rich vegetable oil. There is an abundance of this hydrogenated form of vitamin K_1 in the American diet, accounting for up to 30% of the total dietary vitamin K_1 consumed (10). Therefore, it is important to determine if dihydro-vitamin K_1 can act as a cofactor for vitamin K-dependent γ-glutamyl carboxylase required for the synthesis of vitamin K-dependent proteins. In 1941, Fieser *et al.* (9) used the chick bioassay to compare the biological activity of dihydro-vitamin K_1, among other naphthoquinone-related compounds, with the parent compound, vitamin K_1. Whereas these authors reported an 8-fold reduction in biological activity associated with the saturation of the 2′,3′ double bond of the side chain, other authors reported greater losses in biological activity (15). If dihydro-vitamin K_1 is biologically active in humans, this hydrogenated form of vitamin K_1 may have an important contribution to vitamin K nutrition and would need to be considered in studies that investigate the role of dietary vitamin K intake on human health.

In this study, the dihydro-vitamin K_1 to vitamin K_1 plasma ratio during the stick margarine phase was 1:5, which was greater than the 1:9 ratio in the diet. In an analysis of the Food and Drug Administration's Total Diet Study (FDA TDS), the estimated vitamin K_1 and dihydro-vitamin K_1 daily intakes for a comparable age and gender group were 79 and 19 μg, respectively (10). Therefore, the intake of dihydro-vitamin K_1 in the stick margarine phase of this study is similar to the amount of dihydro-vitamin K_1 normally consumed in the American diet as estimated by the FDA TDS. However, the dietary intakes of vitamin K_1 were ~2.5 times greater than both the current RDA for vitamin K (11) and the estimated intakes from the FDA TDS. Therefore, the higher ratio of dihydro-vitamin K_1 to vitamin K_1 in the plasma as compared to the ratio in the diet may be indicative of either greater absorption or slower clearance of dihydro-vitamin K_1 than vitamin K_1.

In an earlier study that established the normal range for plasma vitamin K_1 (16), dihydro-vitamin K_1 was used as an internal standard so it was not possible to determine the plasma concentrations of endogenous dihydro-vitamin K_1 in these free-living individuals. It is also not known the extent to which earlier studies underestimated circulating vitamin K_1 concentrations, which were based on the direct comparison of peak area ratios of the plasma samples to authentic standards of vitamin K_1 and the corresponding internal standard, dihydro-vitamin K_1. However, continuous modifications to the original assay developed in our laboratory (13) have increased the sensitivity of detection by 3–4-fold. It was only with this enhanced detection that we were able to measure dihydro-vitamin K_1 in plasma of individuals consuming diets that contained vitamin K_1-rich hydrogenated oils. Intake data from the FDA TDS suggest that dihydro-vitamin K_1 is abundant in the American diet, and since it is now known that this compound is absorbed from the diet, the internal standard $K_{1(25)}$ is now used for vitamin K_1, dihydro-vitamin K_1, and vitamin K_1–epoxide assays in our laboratory.

In summary, 2′,3′-dihydro-vitamin K_1 has recently been identified as a dietary form of vitamin K produced during the hydrogenation of vitamin K_1-rich vegetable oils (7). In this study, dihydro-vitamin K_1 was measured in plasma following ingestion of known amounts of a hydrogenated vegetable oil. Dietary intake data from national food consumption surveys indicate that this hydrogenated form of vitamin K_1 is abundant in the American diet (10). Therefore, it is now important to determine the biological activity of dihydro-vitamin K_1 in humans relative to its parent compound, vitamin K_1.

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Dihydro-Vitamin K_1: Primary Food Sources and Estimated Dietary Intakes in the American Diet

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ABSTRACT: Dihydro-vitamin K_1 was recently identified as a dietary form of vitamin K produced during the hydrogenation of vitamin K_1-rich vegetable oils. Dihydro-vitamin K_1 is absorbed, with measurable levels in human plasma following dietary intake. To determine the primary food sources of dihydro-vitamin K_1 in the American diet, 261 foods from the U.S. Food and Drug Administration's (FDA) Total Diet Study (TDS) were analyzed by high-performance liquid chromatography. Of these foods, 36 contained dihydro-vitamin K_1. Fast-food items that were otherwise poor sources of vitamin K_1, such as french fries and fried chicken, contained appreciable amounts of dihydro-vitamin K_1 (36 and 18 μg/100 g, respectively). These nutrient values were then applied to the FDA TDS consumption model to determine average dietary intake of dihydro-vitamin K_1 in 14 age–gender groups. With the exception of infants, all age–gender groups had estimated mean daily dihydro-vitamin K_1 intakes of 12–24 μg, compared to mean daily vitamin K_1 intakes of 24–86 μg. The vitamin K_1 and dihydro-vitamin K_1 intakes were summed, and the dietary contribution of dihydro-vitamin K_1 was expressed as a percentage of total vitamin K intake. Children reported the highest intakes of dihydro-vitamin K_1 (30% of total vitamin K intake), followed by a progressive decrease in percentage contribution with age. There are currently no data on the relative bioavailability of dihydro-vitamin K_1 but, given its abundance in the American diet, this hydrogenated form of vitamin K warrants further investigation.
Lipids 31, 715–720 (1996).

The relationship between the dietary intake of vitamin K_1 and vitamin K status has been inadequately studied because of the limited amount of food composition data available (1). It also has been assumed that a healthy population is not at risk of dietary vitamin K deficiency (2). The current Recommended Dietary Allowance (RDA) for vitamin K is currently set at 80 and 65 μg for adult men and women, respectively (3). This dietary recommendation was established on the assumption that optimal vitamin K nutriture could be determined by the amount of vitamin K required to maintain blood clotting times (4). However, with the understanding that the function of vitamin K is not limited to its role in hemostasis, more sensitive methods for assessing vitamin K nutritional status have been developed by using direct measurements of vitamin K and vitamin K-dependent proteins in blood. Recent studies indicate that the current RDA does not fully support the carboxylation of γ-carboxyglutamic acid residues in extra-hepatic vitamin K-dependent proteins, such as osteocalcin (5,6).

The use of high-performance liquid chromatography (HPLC) has facilitated the routine determination of vitamin K_1 concentrations in common foods and, in the most recent provisional table for vitamin K_1, only food composition data derived exclusively from HPLC analyses were used (7). Vitamin K_1 (phylloquinone) is considered the predominant form of vitamin K found in the diet (8). One of the outstanding characteristics of the provisional table was the overall absence of data on processed foods and mixed dishes.

We recently described HPLC preparation procedures for the determination of vitamin K_1 in various food matrices that were selected to represent foods commonly consumed in the United States (9). In the analysis of foods containing hydrogenated oils, we detected a peak that corresponded to 2′,3′-dihydro-vitamin K_1, a hydrogenated form of vitamin K. We have since confirmed the identity of dihydro-vitamin K_1 using gas chromatography/mass spectrophotometry, and its derivation from vitamin K_1 during the process of hydrogenation (10). Furthermore, we have measured dihydro-vitamin K_1 in human plasma following dietary intake of this compound in the form of margarine (11).

In this article, we present data on the dihydro-vitamin K_1 content in foods obtained from the U.S. Food and Drug Administration's (FDA) Total Diet Study (TDS). In the FDA TDS, more than 250 core foods in the American food supply, representing foods consumed in the United States, were purchased from retail markets four times per year from different geographic regions (12). These food values were then applied to the FDA TDS consumption model for 14 age–gender groups, which was based on data from the United States Department of Agriculture's (USDA) 1987/1988 Nationwide Food Consumption Survey (NFCS) (13). Analysis of these

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Abbreviations: FDA, U.S. Food and Drug Administration; HPLC, high-performance liquid chromatography; $K_{1(25)}$, 2-methyl-3-(3,7,11,15,19-pentamethyl-2-eicosenyl)-1,4-napthalenedione; NFCS, Nationwide Food Consumption Survey; RDA, Recommended Dietary Allowance; SPE, solid-phase extraction; TDS, Total Diet Study; USDA, United States Department of Agriculture.

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foods have identified food classes that are potential contributors to dihydro-vitamin K_1 intake in the United States, and provide dietary intake estimates of this hydrogenated form of vitamin K_1 by 14 age–gender groups.

EXPERIMENTAL PROCEDURES

Collection and preparation of foods. The 1990 FDA TDS food list identified core foods in the American diet on the basis of data from the USDA 1987/1988 NFCS (12). Individual foods and ingredients are collected from four sites per year in the United States and sent to the FDA Field Office Laboratory (Kansas City, MO) for preparation and processing according to instructions described in detail by Pennington (14). For this study, 261 foods were selected from the August 1993 TDS collection for vitamin K_1 and dihydro-vitamin K_1 determination. Each food was homogenized and a 10-g portion was packed in domestic quality ziplock-type plastic bags. The 10-g portions were frozen, packed with frozen ice packs, and shipped by air to the Vitamin K Laboratory at the Jean Mayer USDA Human Nutrition Center at Tufts University, where they were stored, protected from light, at –20°C until analysis.

Vitamin K_1 and dihydro-vitamin K_1 determination in foods. Each homogenate was analyzed in triplicate for vitamin K_1 and dihydro-vitamin K_1 by an HPLC method as described elsewhere (9). Briefly, 0.25–0.50 g aliquots of each homogenate were weighed directly into 50-mL polypropylene centrifuge tubes (Corning Co., Corning, NY). An appropriate amount of the internal standard, vitamin 2-methyl-3-(3,7,11,15,19-pentamethyl-2-eicosenyl)-1,4-napthalenedione [$K_{(1,25)}$], was added directly to the test portions. Fifteen milliliters of 2-propanol/hexane (3:2, vol/vol) and 4 mL of H_2O were added, followed by sonification using a Branson Model 350 sonifer-cell disrupter (Branson Ultrasonics Corp., Danbury, CT). The mixtures were then vortexed (10 min), and centrifuged at 1000 × *g* (10 min). After phase separation, the upper hexane layer was aspirated into a 16 × 100 culture tube, evaporated to dryness under reduced pressure in a centrifugal evaporator (Savant Instrument Inc, Farmingdale, NY), and the residue was redissolved in 4 mL of hexane. An aliquot of the reconstituted lipid extract was applied to a 6-mL solid-phase extraction (SPE) silica column (J.T. Baker Inc., Chicago, IL) which had been preconditioned by successive washes of 8 mL of hexane/diethyl ether (93:3, vol/vol), followed by 8 mL of hexane. The SPE silica column was then washed with an additional 8 mL of hexane to remove the hydrocarbons. The fraction containing vitamin K_1 was eluted with 8 mL of hexane/diethyl ether (97:3, vol/vol), collected, and evaporated to dryness.

To further purify foods with a high lipid content, such as meat, butter, and margarine, a reversed-phase C_{18} SPE extraction was used. After evaporation, the residue from the hexane–ether eluate was dissolved in 200 µL of 2-propanol by heating (45°C) for 10 min. The reversed-phase C_{18} columns (6 mL; J.T. Baker) were preconditioned by successive washes with 6 mL of methanol/methylene chloride (80:20, vol/vol), followed by 6 mL of 100% methanol and 6 mL of 100% H_2O. The dissolved residue was applied directly to the preconditioned packing. The column was washed with 6 mL of methanol/H_2O (95:5, vol/vol), followed by 6 mL of 100% acetonitrile, and the analyte was eluted from the column with 10 mL of methanol/methylene chloride (80:20, vol/vol), collected, and evaporated to dryness. The final residue was reconstituted initially in 30 µL of 100% methylene chloride, followed by 270 µL of methanol containing 10 mM zinc chloride, 5 mM acetic acid, and 5 mM sodium acetate. A 150-µL aliquot was injected into the HPLC.

Quantitation of vitamin K_1 and dihydro-vitamin K_1 in the homogenates was performed by direct comparison of peak area ratios of the foods to authentic standards of vitamin K_1, dihydro-vitamin K_1, and vitamin $K_{1(25)}$, using a Waters 860 chromatography data system (Waters Chromatography, Milford, MA). The lower limit of detection was set at 0.01 µg of vitamin K_1 and/or dihydro-vitamin K_1/100 g of food. Vitamin $K_{1(25)}$, the internal standard that was used for all the FDA TDS analyses, was a gift from Hoffman-La Roche and Co. (Basel, Switzerland). All foods were analyzed within seven months of collection and preparation. The vitamin K_1 concentrations of the 261 homogenates have been reported elsewhere (15).

Survey design. The 1990 revision of the FDA TDS was based on data from the 1987–1988 NFCS, which was a nationwide area probability sample of the population in 48 coterminous states (12). Food intake data were collected over four seasons, with a different sample of participants in each season (16). Individual dietary data consisted of a single 24-h recall and a 2-d food record collected on consecutive days.

Fourteen age–gender groups were selected for the 1990 revision of the TDS, as described in detail by Pennington (13). Of the NFCS participants who were within the selected 14 age–gender groups (total 7,891), the TDS only included those participants from whom three days of dietary data were available (total 3,634). Although this exclusion of participants may have created an additional response bias, it is assumed that an increase in the days of dietary recording by each participant will reduce the impact of intraindividual random variation in dietary intake (17).

Estimation of vitamin K_1 and dihydro-vitamin K_1 intakes. As described in detail elsewhere, 6,148 food codes, food names, and food descriptions used in the 1987–1988 NFCS were divided among 14 major categories (13). These foods were then manually aggregated into groups (total 261) within these 14 categories on the basis of similarity of food type, use in the diet, preparation methods, and composition.

The vitamin K_1 and dihydro-vitamin K_1 values were matched with corresponding food items in the TDS, and the daily intakes of each food were calculated for each of the 14 age–gender categories as described by Pennington (13). The daily intakes of vitamin K_1 and dihydro-vitamin K_1 were specified in µg/d and as a percentage of total daily µg intake. The results of the analyses of phylloquinone in the TDS have been reported elsewhere (18).

RESULTS

Food composition. Of the 261 foods analyzed in the FDA TDS August 1993 collection for vitamin K, 36 foods contained dihydro-vitamin K_1 in concentrations greater than 1.0 μg/100 g of food (Table 1). All 36 foods contained hydrogenated fats or oils, with the highest concentrations of dihydro-vitamin K_1 measured in assorted cookies and stick margarine. However, when expressed in μg per average serving size, the richest sources of dihydro-vitamin K_1 were commercial apple pie (27 μg), followed by fast-food french fries (25 μg), and baked biscuits from refrigerated dough (21 μg).

Foods that are otherwise poor dietary sources of vitamin K_1, such as root vegetables and animal products (7,15), can contribute to overall vitamin K nutriture when processed with vitamin K_1-rich vegetable oils, as demonstrated for potatoes and chicken (Figs. 1 and 2). The vitamin K_1 concentrations of roasted chicken and homemade fried chicken measured in the August 1993 FDA TDS collection were <0.001 and 3.8 μg/100 g of chicken, respectively (15). Fast-food items, such as french fries and chicken nuggets, had large concentrations of dihydro-vitamin K_1 relative to their vitamin K_1 contents (Table 1).

Dietary intake. The total mean dihydro-vitamin K_1 intake reported for the infants was 1 μg/d, which accounted for ~1% of the total vitamin K intake (Table 2). This was an expected

TABLE 1
Primary Food Sources of Dihydro-Vitamin K_1 in the American Diet: Data from the August 1993 Collection of the FDA TDS[a]

Food description	Average serving (g)	Vitamin K_1		Dihydro-vitamin K_1	
		μg/100 g of food	μg/serving	μg/100 g of food	μg/serving
Meat, poultry, and fish					
Chicken nuggets, fast-food	109	1.5	1.6	9.4	10
Chicken, fried, fast-food	85	1.3	1.1	18	20
Fish sticks, frozen, heated	85	6.8	5.8	8.2	7.0
Grain products					
Biscuit, refrigerated dough, baked	70	4.6	3.2	30	21
Tortilla, flour	70	3.1	2.2	6.5	4.6
Blueberry muffin commercial	57	25	14	3.9	2.2
Graham crackers	28	8.9	0.5	21	6.0
Saltine crackers	30	3.6	1.1	21	6.4
Butter-type crackers	30	13	3.9	59	18
Vegetables					
French fries, frozen, heated	70	7.1	5.0	9.1	6.4
French fries, fast-food	68	4.4	3.0	36	25
Mashed potatoes, from flakes[b]	105	5.1	5.4	5.2	5.5
Coleslaw with dressing, homemade	120	100	119	12	14
Mixed dishes and meals					
Chicken pot pie	277	2.7	7.5	1.4	3.9
Tuna noodle casserole	240	20	48	3.4	8.2
Frozen meal—turkey	312	5.3	17	3.4	11
Macaroni and cheese, from instant mix	200	5.3	11	6.3	13
Egg, cheese, ham muffin, fast-food	16	3.7	5.4	6.2	9.1
Tacos/tostadas, fast-food	171	16	28	4.7	8.0
Fish sandwich, fast-food	158	17	26	4.8	7.6
Desserts					
Chocolate cake with chocolate icing	64	13	8.4	15	9.8
Yellow cake with yellow icing	64	8.5	5.4	4.6	2.9
Sweet roll/danish, commercial	65	11	7.3	25	17
Cake doughnuts with icing	47	9.8	4.6	31	15
Brownies, commercial	57	14	8.0	16	8.9
Sugar cookies	30	11	3.4	49	15
Chocolate chip cookies, commercial	30	10	3.0	45	14
Sandwich cookies, commercial	30	8.7	2.6	38	11
Apple pie, fresh/frozen, commercial	125	11	14	22	27
Pumpkin pie, fresh/frozen, commercial	125	10	11	18	16
Snacks					
Corn chips	28	7.3	2.0	6.9	1.9
Pretzels	28	2.9	0.8	1.3	0.5
Fats and dressings					
White sauce, homemade	66	6.9	4.6	7.4	4.9
Mayonnaise, regular	14	41	5.7	42	5.9
Margarine, stick	14	33	4.6	57	8.0
Cream substitute, frozen	15	5.7	0.9	7.5	1.1

[a]FDA TDS, U.S. Food and Drug Administration Total Diet Study. [b]Whole milk and margarine were added according to package instructions (Ref. 14).

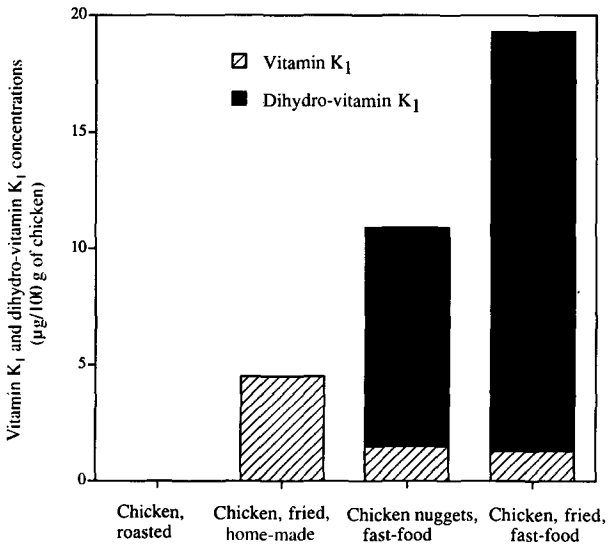


FIG. 1. The effects of processing on the vitamin K_1 and dihydro-vitamin K_1 concentrations of chicken. Vitamin K_1 data for roasted, homemade, and fried chicken were obtained from Booth *et al.* (Ref. 15). Homemade chicken was fried in a vegetable oil blend.

result because 87% of total dietary vitamin K_1 intake for this age group is attributed to infant formula (18), which contains no dihydro-vitamin K_1. In contrast, the estimated mean dihydro-vitamin K_1 intake for the two-year-old group was 12 μg/d, which accounted for 33% of the total vitamin K intake, assuming equivalent bioavailability of dihydro-vitamin K_1 compared to vitamin K_1. The five primary dietary sources of dihydro-vitamin K_1 for this age group were fast-food french fries, sugar cookies, instant mashed potatoes, margarine, and chocolate chip cookies (19, 9, 7, 7, and 6% of total dihydro-vitamin K_1 intake, respectively). The percentage contribution of dihydro-vitamin K_1 to total vitamin K intake was greatest among the two-year-old children, with a progressive decrease in percentage contribution with age. There was a higher percentage contribution of dihydro-vitamin K_1 to total dietary vitamin K intake among men when compared to the women. Among the adults, the five primary food sources of dihydro-vitamin K_1 were fast-food french fries, margarine, mayonnaise, heated french fries, and macaroni and cheese prepared from a package, each of which contributed 5% or less of the total vitamin K intake.

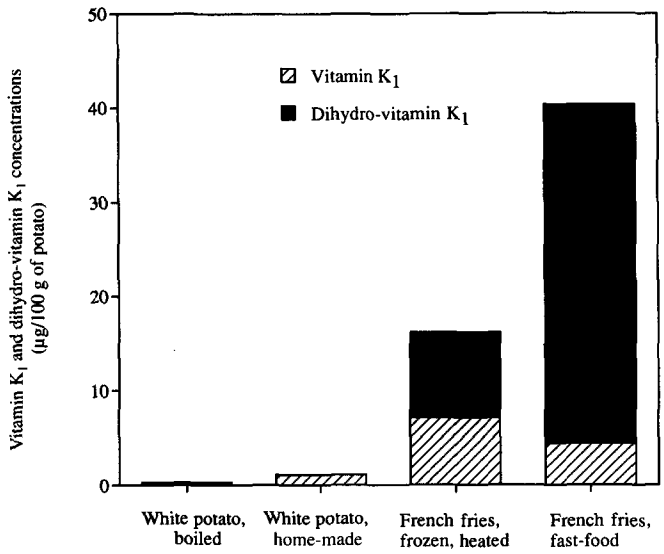


FIG. 2. The effects of processing on the vitamin K_1 and dihydro-vitamin K_1 concentrations of potatoes. Vitamin K_1 data for boiled and baked potatoes were obtained from Booth *et al.* (Ref. 15).

TABLE 2
The Contribution of Dihydro-Vitamin K_1 to Total Dietary Vitamin K Intake: Data from the 1990 FDA TDS[a]

Group	Number[b]	RDA[c]	K_1 intake (μg)	Dihydro-K_1 intake (μg)	Total[d] (μg)
Infants					
6-mon infants	141	10	77 (99%)[e]	1 (1%)	78
Children					
2-yr	152	15	24 (67%)	12 (33%)	36
6-yr	154	20	46 (72%)	18 (28%)	64
10-yr	119	30	45 (70%)	19 (30%)	64
14–16-yr (boys)	174	45–65	64 (74%)	23 (26%)	87
14–16-yr (girls)	188	45–55	52 (71 %)	21 (29%)	73
Younger adults					
25–30-yr (men)	386	80	66 (73%)	24 (27%)	90
25–30-yr (women)	492	65	59 (78%)	17 (22%)	76
40–45-yr (men)	293	80	86 (79%)	23 (21%)	109
40–45-yr (women)	319	65	71 (82%)	16 (18%)	87
Older adults					
60–65-yr (men)	238	80	80 (81%)	19 (19%)	99
60–65-yr (women)	313	65	76 (82%)	17 (18%)	93
70+-yr (men)	263	80	80 (80%)	21 (21%)	101
70+-yr (women)	402	65	82 (84%)	15 (16%)	97

[a]See Table 1 for abbreviations.
[b]The number of subjects as stratified by age and/or gender.
[c]Reference 3.
[d]Summation of vitamin K_1 and dihydro-vitamin K_1, assuming equivalent biological activity of dihydro-vitamin K_1 with vitamin K_1.
[e]Numbers in parentheses are the percentages of the total intake of both vitamin K_1 and dihydro-vitamin K_1.

DISCUSSION

We have recently demonstrated that the hydrogenation of vitamin K_1-rich vegetable oils converts vitamin K_1 to dihydro-vitamin K_1, with an overall loss of the vitamin that is proportional to the degree of hydrogenation (10). The data presented in this study identify those food classes that are most affected by the impact of hydrogenation on the vitamin K_1 content of the diet. Foods that are otherwise poor sources of vitamin K_1, such as the root vegetables and animal meats, have a significant dihydro-vitamin K_1 content when they are processed using partially hydrogenated vitamin K_1-rich vegetable oils, such as soybean, canola, and cottonseed. There is also a small increase in the vitamin K_1 content of these processed foods because partial hydrogenation of vitamin K_1-rich oils does not completely convert all the vitamin K_1 to dihydro-vitamin K_1.

These are the first available dihydro-vitamin K_1 food composition data for commonly-consumed foods in the American diet. Given that the dihydro-vitamin K_1 and vitamin K_1 values presented in this study were derived from a single TDS collection, the FDA TDS foods do not reflect the regional and seasonal differences in the phylloquinone content of plant oils (19,20), nor do they reflect the variation in dihydro-vitamin K_1 content associated with conditions of hydrogenation (10). Therefore, the dihydro-vitamin K_1 values associated with the FDA TDS lack the sensitivity to reflect the range of individual intakes. Despite the limitations of the FDA TDS model, this study does indicate that dihydro-vitamin K_1 is abundant in the average American diet.

Dihydro-vitamin K_1 is absorbed from a dietary source, such as margarine, as indicated by corresponding peaks measured in human plasma by HPLC following consumption of known amounts of dihydro-vitamin K_1 (11). If there is an equivalent bioavailability of dihydro-vitamin K_1 compared with vitamin K_1, the intake data presented in this study would have important implications for several population groups. Although in the FDA TDS model, children had estimated mean vitamin K_1 intakes equivalent or above the current RDA for their respective age groups (18), the amount of dihydro-vitamin K_1 in the diet would increase the absolute intake of vitamin K by 30% if this hydrogenated form of vitamin K is indeed bioavailable. In the FDA TDS model, the 25–30-year-old adults had estimated mean dietary vitamin K_1 intakes lower than the current RDA of 65–80 μg of vitamin K_1 (3). The addition of dihydro-vitamin K_1 to the total dietary intake of vitamin K shifted intakes above the current RDA, assuming equivalent bioavailability. This is particularly critical because recent studies among young and older adults indicate that the current RDA for vitamin K does not sufficiently support full carboxylation of the γ-carboxyglutamic acid in extra-hepatic vitamin K-dependent proteins, such as osteocalcin (5,6).

Of concern is the high-fat content of those foods that are rich sources of dihydro-vitamin K_1, such as margarine, high-fat desserts, high-fat potatoes, and high-fat grain based dishes. Other foods, such as leafy vegetables, are excellent dietary sources of vitamin K_1 and should be promoted if the goal is to increase dietary intake of vitamin K_1. However, this study has demonstrated that this hydrogenated form of vitamin K is present in 36 of the 261 most commonly-consumed foods in the American diet, and the estimated dietary intakes could account for as much as 30% of the total vitamin K intake in certain age groups. A comparison of dietary practices among older Americans indicated that there have been only minor changes in fat consumption from foods such as high-fat desserts, between 1977–1978 and 1987–1988 (16). Furthermore, younger women have been identified as the age group among women that are consuming more meals away from home, with higher intakes of total and saturated fat associated with fast-food consumption (21). Therefore, the abundance of 2′,3′-dihydro-vitamin K_1 in the American diet warrants further investigation into its biological activity as consumption could increase otherwise low current vitamin K intake estimates if this hydrogenated form has equivalent biological activity to the parent compound, vitamin K_1. Conversely, if 2′,3′-dihydro-vitamin K_1 does not contribute to vitamin K nutriture, commercial hydrogenation of vitamin K_1-rich vegetable oils may be reducing the already low vitamin K_1 intakes reported in the FDA TDS.

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Prevention of Cholesterol Cholelithiasis by Dietary Unsaturated Fats in Hormone-Treated Female Hamsters

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ABSTRACT: We examined the effect of diet on gallstone incidence and the composition of biliary phosphatidylcholines in methyltestosterone-treated female hamsters. These hamsters were fed a nutritionally adequate purified lithogenic diet containing 2% corn oil, 4% butterfat, 0.3% cholesterol, and 0.05% methyltestosterone, resulting in a cholesterol gallstone incidence of 86%. This incidence was lowered when mono- and polyunsaturated fats or fatty acids were added to the diet: 2.5% oleic acid resulted in total prevention of cholesterol cholelithiasis, 2.5% linoleic acid, and 4% safflower oil (78% linoleic acid content) reduced gallstone incidence to 26 and 8%, respectively. An additional 4% butterfat (29% oleic acid content) produced gallstones in 50% of the animals. At the end of the 6-wk feeding period, the bile of all hamsters was supersaturated with cholesterol. The major biliary phosphatidylcholine species in all groups were (*sn*-1–*sn*-2): 16:0-18:2, 16:0-18:1, 18:0-18:2, 16:0-20:4, and 18:2-18:2. The safflower oil- and linoleic acid-fed hamsters exhibited an enrichment of 16:0-18:2 (16–18%); added butterfat or oleic acid increased the proportion of 16:0-18:1 (9 and 25%, respectively). We conclude that the phosphatidylcholine molecular species in female hamster bile can be altered by dietary fats/fatty acids and that mono- and polyunsaturated fatty acids play a role in suppressing the induced cholelithiasis.

Lipids 31, 721–727 (1996).

We previously employed male Sasco hamsters as a model of cholesterol cholelithiasis (1). In this model, gallstone induction was accomplished with a nutritionally adequate purified diet which contained 4% butterfat and 0.3% cholesterol. Female hamsters from the same source did not develop supersaturated bile or gallstones when fed the standard lithogenic diet (LD) (2). However, when the synthetic androgen, methyltestosterone (MeT), was added to the LD, female hamsters developed cholesterol gallstones at the same rate as the males (3). We previously showed that stone incidence in the male hamster was modulated by the specific fats or fatty acids in the experimental diet: saturated fats and fatty acids (palmitic acid) enhanced gallstone formation while unsaturated fats (safflower oil, corn oil, olive oil) had the opposite effect (4–9). It was not known whether stone incidence in hormone-treated female hamsters was affected by the type of dietary fat in a similar manner.

Biliary components are believed to effect cholelithiasis. Attempts to correlate cholesterol gallstone formation in humans and experimental animals with modifications of the molecular species of biliary phosphatidylcholines (PC) have yielded conflicting data and interpretations (10–21). A number of investigators have reported the total biliary fatty acid composition of hamsters fed different diets ranging from Dam's essential fatty acid-deficient diet to nutritionally adequate diets with defined fatty acid composition (13,22–24). In none of these studies was it attempted, however, to analyze individual PC species. We previously found that in male hamsters, dietary fat affected the distribution of biliary cholesterol between vesicles and micelles (9). We now report experiments in hormone-treated female hamsters describing the correlation between dietary fatty acid composition, gallstone incidence, and biliary PC species.

MATERIALS AND METHODS

Animals and diet. Four-week-old (50–60 g) female golden Syrian hamsters (*Mesocricetus auratus*) were obtained from Sasco Inc. (Omaha, NE). The hamsters were fed a pelleted nutritionally adequate purified LD (Dyets, Inc., Bethlehem, PA) containing 0.3% cholesterol and 0.05% MeT with supplementation of specific unsaturated fats or fatty acids for 6 wk. The composition of the LD was as follows: 43.4% cornstarch, 20.0% casein, 14.6% dyetrose (soluble starch), 10.0% fiber (cellulose), 5.0% salt mix (modified U.S.P. XIV salt mix no. 200951), 4.0% butterfat, 2.0% corn oil, 0.5% vitamin mix (no. 300000), 0.3% cholesterol, 0.2% choline chloride, and 0.05% Met. The hamsters were placed into one of five groups: group 1 [22 hamsters], LD; group 2 [23 hamsters], LD + 2.5% linoleic acid (18:2); group 3 [24 hamsters], LD + 2.5% oleic acid (18:1); group 4 [26 hamsters], LD + 4.0% butterfat;

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Abbreviations: CSI, cholesterol saturation index; GLC, gas–liquid chromatography; HI, hydrophobicity index; HPLC, high-performance liquid chromatography; LD, lithogenic diet; 18:2, linoleic acid; MeT, methyltestosterone; 18:1, oleic acid; PC, phosphatidylcholine; PC(16:1-16:1), 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine; PC(16:0-20:4), 1-palmitoyl-2-arachidonoyl; PC(16:0-18:2), 1-palmitoyl-2-linoleoyl; PC(18:0-18:2), 1-stearoyl-2-linoleoyl; PC(16:0-18:1), 1-palmitoyl-2-oleoyl.

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group 5 [24 hamsters], LD + 4.0% safflower oil. At the end of the feeding period, the gallbladders were examined for gallstones and cholesterol crystals. Cholesterol gallstones could be seen through the gallbladder wall and were between 1–5 mm in diameter. The bile (30–50 μL per hamster) was removed for determination of the biliary lipid composition and the major PC molecular species. The gallstones as well as an aliquot of bile was examined under polarized light microscopy using an Olympus MCHAP light microscope as described earlier (4,6) (Olympus Corp., Lake Success, NY).

Analytical procedures. Liver and plasma cholesterol levels were determined as described earlier (25). Biliary bile acids were determined as the methyl ester acetates by gas–liquid chromatography (GLC) (26). Total biliary phospholipids were measured using the Wako phospholipid B kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Cholesterol concentrations in bile were determined by GLC as previously described (26). Gallstones were analyzed by diffuse reflectance Fourier transform infrared spectroscopy (27).

The molecular species of PC in bile were determined as the benzoyl diacylglycerol derivatives by reverse-phase high-performance liquid chromatography (HPLC). The PC was isolated by HPLC from pooled hamster bile (50 μL) using a LiChrospher Si-100 column (25 cm × 4.0 mm, 5 μ particle size; E. Merck, Darmstadt, Germany) according to the method of Patton *et al.* (28). The PC fraction was hydrolyzed to diglycerides with phospholipase C Type IV from *Bacillus cereus* (Sigma Chemical Co., St. Louis, MO) and then derivatized using benzoyl chloride as described by Patton *et al.* (29) and Ullman and McCluer (30). Analysis of individual PC was carried out by HPLC on a 5 μ Ultrasphere C-18 column (250 mm × 4.6 mm; Beckman Instruments, Fullerton, CA). The fatty acids of the individual PC were then isolated and identified after formation of the methyl esters (Meth Prep II; Alltech Associates Inc., Deerfield, IL) by capillary gas chromatography on a Supelcowax fused silica capillary column (30 m, 0.32 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, PA). The amount of cholesterol present in biliary cholesterol–phospholipid vesicles and micelles was carried out as previously described (5).

Reference compounds. PC used as standards were purchased from Avanti Polar-Lipids, Inc. (Alabaster, AL). 1,2-Dipalmitoleoyl-*sn*-glycero-3-phosphocholine [PC(16:1-16:1)] was used as an internal standard. 1-Palmitoyl-2-arachidonoyl [PC(16:0-20:4)], 1-palmitoyl-2-linoleoyl [PC(16:0-18:2)], 1-stearoyl-2-linoleoyl [PC(18:0-18:2)], and 1-palmitoyl-2-oleoyl [PC(16:0-18:1)] were also purchased from Avanti for use as standards. Individual fatty acid methyl ester standards were purchased from Alltech.

Calculations and statistics. The cholesterol saturation indices (CSI) were determined using previously published methods (31,32). The total bile acid and PC molecular species hydrophobicity indices (HI) were determined using the relative retention times obtained by GLC and HPLC, respectively, as described earlier (33,34). All data were reported as mean ± SD. Differences between groups were calculated using analysis of variance to determine the F statistic. Student's *t*-test was then used for values for which the F statistic was significant ($P < 0.05$) (35).

RESULTS

As depicted in Table 1, butterfat, the major fat in the standard LD, contains mostly palmitic (16:0, 27%) and oleic (18:1, 29%) acids. Safflower oil contains mostly linoleic acid (18:2, 78%).

Liver and plasma cholesterol levels are summarized in Table 2. Liver cholesterol in the LD diet + 4% butterfat group 4 (77 mg/g) was significantly lower than the other experimental animals (range, 77–109 mg/g); in contrast, plasma cholesterol levels (406 mg/dL) in this group were significantly higher compared to the other experimental groups (range, 318–406 mg/dL).

Table 3 summarizes the incidence of gallstones as a function of different fats/fatty acids in the diet. The control LD (group 1) had a gallstone incidence of 86% which was significantly reduced by the fatty acids, 18:2 (26%, group 2) and 18:1 (0%, group 3). Gallstone incidence was also reduced by added fats, 4% butterfat (50%, group 4) and 4% safflower oil (8%, group 5). Table 3 also shows the biliary lipid composition. Total lipids were similar, ranging from 9.32 to 11.32 g/dL. The CSI was lowest in the 18:2- and safflower oil-fed groups (96% for both groups 2 and 5). All other hamsters had a CSI greater than unity. The mole % phospholipids ranged from 16.10 to 18.38%; the mole % cholesterol in the 18:2- and safflower oil-fed hamsters was lower than in the controls (5.77 and 5.75%, groups 2 and 5, respectively vs. 7.04%, group 1). The amount of cholesterol present in biliary cholesterol–phospholipid vesicles ranged from 58% (group 4) to 70% (group 2).

Table 4 summarizes the bile acid composition of hamster gallbladder bile. Cholic acid was lowered by 18:2 (37.84%,

TABLE 1
The Major Fatty Acids (%) in Butterfat and Safflower Oil[a]

	Fatty acid											
Fat	4:0	6:0	8:0	10:0	12:0	14:0	16:0	18:0	16:1	18:1	18:2	18:3
Butterfat	4	2	1	3	3	11	27	12	2	29	2	1
Safflower oil	0	0	0	0	0	0	7	2	0	13	78	0

[a]4:0, Butyric; 6:0, capric; 8:0, caprylic; 10:0, caproic; 12:0, lauric; 14:0, myristic; 16:0, palmitic; 18:0, stearic; 16:1, palmitoleic, 18:1, oleic; 18:2, linoleic; 18:3, linolenic. Butterfat also contains 15:0 and 17:0 at about 3% of total fatty acids. Source: Reference 59.

TABLE 2
Effect of Fat/Fatty Acid Feeding on Liver and Plasma Cholesterol[a]

Group	Diet	Liver cholesterol (mg/g)	Plasma cholesterol (mg/dl)
1	LD (control)	90 ± 9	318 ± 50
2	LD + 2.5% linoleic acid	99 ± 13	337 ± 34
3	LD + 2.5% oleic acid	109 ± 11	336 ± 69
4	LD + 4.0% butterfat	77 ± 13a,b	406 ± 70c,d
5	LD + 4.0% safflower oil	96 ± 9	343 ± 36

[a]Numbers are mean ± SD; see the Materials and Methods section for composition of the LD (lithogenic diet). a, Differs from groups 2, 3, and 5, $P < 0.001$; b, differs from group 1, $P < 0.003$; c, differs from groups 1,2, and 5, $P < 0.001$; d, differs from group 3, $P < 0.002$.

TABLE 3
Effect of Fat/Fatty Acid Feeding on Cholesterol Gallstone Incidence and Biliary Lipids[a]

Group	Diet	Cholesterol gallstones (%)	Phospholipids (mol%)	Cholesterol (mol%)	Bile acids (mol%)	Total lipids (g/dL)	Cholesterol saturation (%)	Vesicular cholesterol (%)
1	LD (control)	19/22 (86) a	18.38 ± 2.39 b	7.04 ± 2.48 c	74.22 ± 4.01 d	10.67 ± 2.41	109 ± 32	57 9 ± 9
2	LD + 2.5% linoleic acid	6/23 (26) e	17.18 ± 2.58	5.77 ± 1.03 f	76.88 ± 3.51	10.14 ± 2.92	96 ± 16 g	70 ± 11
3	LD + 2.5% oleic acid	0/24 (0) h	17.26 ± 2.28	6.67 ± 1.54 i	76.07 ± 4.08	9.32 ± 2.14 j	111 ± 13 j	69 ± 4
4	LD + 4.0% butterfat	13/26 (50) k	16.10 ± 2.28	6.50 ± 1.95	77.30 ± 3.86	10.44 ± 1.54	113 ± 27 l	58 ± 8
5	LD + 4.0% safflower oil	2/24 (8)	16.91 ± 2.23	5.75 ± 1.58	77.34 ± 3.42	11.32 ± 2.43	96 ± 22	69 ± 3

[a]Numbers are mean ± SD; see the Materials and Methods section for composition of the LD (lithogenic diet). Gallstone differences determined by chi-squared; all others determined by *t*-test with f-test of SD. a, Differs from groups 2 and 3, $P < 0.005$; group 4, $0.005 < P < 0.01$; group 5, $P < 0.05$; b, differs from group 4, $P < 0.01$; group 5, $P < 0.04$; c, differs from group 2, $P < 0.04$; group 5, $P < 0.05$; d, differs from group 2, $P < 0.03$; groups 4 and 5, $P < 0.01$; e, differs from group 3, $0.005 < P < 0.01$; f, differs from group 3, $P < 0.03$. g, differs from groups 3 and 4, $P < 0.01$; h, differs from group 4, $P < 0.005$; i, differs from group 5, $P < 0.05$; j, differs from group 5, $P < 0.01$; k, differs from group 5, $P < 0.005$; l, differs from group 5, $P < 0.02$.

group 2), 18:1 (36.13%, group 3), and butterfat (37.72%, group 4) as compared to the control diet (41.79%, group 1). The 18:1-fed hamsters had less hyodeoxycholic acid (7.84%, group 3) as compared to the 18:2 (10.23%, group 2), butterfat (9.97%, group 4), and safflower oil (9.48%, group 5). The HI were similar in all groups.

Table 5 summarizes the major PC molecular species in hamster bile. The major species present were (*sn*-1–*sn*-2): 16:0-18:2, 16:0-18:1, 18:0-18:2, 16:0-20:4, and 18:2-18:2, The four minor species: 16:1-18:2, 18:1-18:2, 18:0-20:4, and 18:1-18:1 comprised less than 10% of PC. Feeding 18:2 and safflower oil increased 16:0-18:2 (54.39%, group 2 and 53.54%, group 5 vs. 46.21%, group 1) and 18:0-18:2 (9.54%, group 2 and 10.27%, group 5 vs. 7.80%, group 1). Feeding 18:1 and butterfat elevated 16:0-18:1 (30.26%, group 3 and 26.47%, group 4 vs. 24.21%, group 1). The HI was lowered by 18:2 and safflower oil (0.19, groups 2 and 5), raised by 18:1 (0.27, group 3), and unaffected by butterfat (0.23, group 4) as compared to the controls (0.23, group 1).

DISCUSSION

We have developed a hamster model of cholesterol cholelithiasis that is responsive to specific fats and/or fatty acids in the diet. Unlike the males, female hamsters form cholesterol gallstones only when the synthetic androgen, MeT is added to the standard LD (2,3). Our LD which contained 4% butterfat and 0.3% cholesterol produced cholesterol gallstones in male

TABLE 4
Effect of Fat/Fatty Acid Feeding on Biliary Bile Acid Composition (%) and Hydrophobicity[a]

Group	Diet	LCA	DCA	CDCA	CA	ACA	HDCA	MDCA	HI
1	LD (control)	2.43 ± 0.55	11.14 ± 3.79	23.84 ± 3.65 a	41.79 ± 1.97 b	3.03 ± 0.88 c	8.13 ± 2.49[d]	2.41 ± 0.82 e	19.47 ± 2.12
2	LD + 2.5% linoleic acid	2.59 ± 1.04	11.04 ± 3.06 f	24.72 ± 5.68	37.84 ± 5.69	2.16 ± 0.63 g	10.23 ± 3.42[h]	3.26 ± 1.33 i	18.55 ± 3.38
3	LD + 2.5% oleic acid	2.35 ± 0.82	10.87 ± 3.40	23.96 ± 4.37 g	36.13 ± 6.69	2.74 ± 0.75 j	7.84 ± 2.45 k	2.47 ± 0.82 l	19.90 ± 4.37
4	LD + 4.0% butterfat	2.10 ± 0.66	9.42 ± 2.35	27.70 ± 7.91	37.72 ± 5.55	1.83 ± 0.47 m	9.97 ± 2.83	2.94 ± 0.78	18.73 ± 4.32
5	LD + 4.0% safflower oil	2.20 ± 0.56	9.24 ± 2.35	26.61 ± 6.41	40.03 ± 7.06	2.78 ± 0.68	9.48 ± 2.35	3.06 ± 0.71	18.19 ± 3.07

[a]Numbers are mean ± SD; see the Materials and Methods section for composition of the LD (lithogenic diet). LCA, lithocholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; CA, cholic acid; ACA, allocholic acid; HDCA, hyodeoxycholic acid; MDCA, murideoxycholic acid; HI, hydrophobicity index. a, Differs from group 4, $P < 0.04$; b, differs from groups 2, 3, and 4, $P < 0.01$; c, differs from groups 2 and 4, $P < 0.01$; d, differs from groups 2 and 4, $P < 0.03$; e, differs from group 2, $P < 0.02$; group 4, $P < 0.03$; group 5, $P < 0.01$; f, differs from group 4, $P < 0.05$; group 5, $P < 0.03$; g, differs from group 4, $P < 0.05$; h, differs from group 3, $P < 0.01$; i, differs from group 3, $P < 0.02$; j, differs from group 4, $P < 0.01$; k, differs from group 4, $P < 0.01$; group 5, $P < 0.03$; l, differs from group 5, $P < 0.02$; m, differs from group 5, $P < 0.01$.

TABLE 5
Effect of Fat/Fatty Acid Feeding on Biliary Phosphatidylcholine Molecular Species (%)[a]

Group	Diet	18:2-18:2	16:0-20:4	16:0-18:2	16:0-18:1	18:0-18:2	Hydrophobicity index
1	LD (control)	2.22 ± 0.39	7.46 ± 0.51 a	46.21 ± 1.26 b	24.21 ± 0.40 c	7.80 ± 0.50 d	0.23 ± 0.02 e
2	LD + 2.5% linoleic acid	1.96 ± 0.34	7.64 ± 1.00	54.39 ± 1.53 f	16.65 ± 0.50 f	9.54 ± 0.76 f	0.19 ± 0.02 f
3	LD + 2.5% oleic acid	2.30 ± 0.32	8.26 ± 0.56	42.73 ± 1.32 g	30.26 ± 2.29 h	7.69 ± 0.67 i	0.27 ± 0.02 g
4	LD + 4.0% butterfat	2.09 ± 0.17	7.85 ± 0.44	46.86 ± 1.97	26.47 ± 1.41 j	6.44 ± 0.13 j	0.23 ± 0.01 j
5	LD + 4.0% safflower oil	2.21 ± 0.12	7.68 ± 0.23	53.54 ± 0.52	16.82 ± 0.34	10.27 ± 0.09	0.19 ± 0.00

[a]Numbers are mean ± SD; see the Materials and Methods section for composition of the LD (lithogenic diet). Phosphatidylcholine (PC) molecular species were determined as benzoyl derivatives by high-performance liquid chromatography. 1-Acyl-2-acyl-*sn*-glycerol-3-phosphocholines: 18:2-18:2, 1-linoleoyl-2-linoleoyl; 16:0-20:4, 1-palmitoyl-2-arachidonoyl; 16:0-18:2, 1-palmitoyl-2-linoleoyl; 16:0-18:1, 1-palmitoyl-2-oleoyl; 18:0-18:2, 1-stearoyl-2-linoleoyl. 16:1-18:2, 18:1-18:2, 18:1-18:1, and 18:0-20:4. The minor species 16:1-18:2, 18:1-18:2, 18:1-18:1, and 18:0-20:4 totalled 7–9% of PC. a, Differs from group 3, $P < 0.05$; b, differs from groups 2, 3, and 5, $P < 0.01$; c, differs from groups 2, 3, and 5, $P < 0.01$; group 4, $P < 0.03$; d, differs from groups 2, 4, and 5, $P < 0.01$; e, differs from groups 2, 3, and 5, $P < 0.02$; f, differs from groups 3 and 4, $P < 0.01$; g, differs from groups 4 and 5, $P < 0.01$; h, differs from groups 4 and 5, $P < 0.02$; i, differs from group 4, $P < 0.02$; group 5, $P < 0.01$; j, differs from group 5, $P < 0.01$.

hamsters that was enhanced by the saturated fatty acid, palmitic acid, and suppressed by unsaturated fats such as safflower, olive, and corn oils (4–9). The mechanism(s) for the effects of different fats is not known but was ascribed to changes in the vesicular/micellar distribution of biliary cholesterol or the molecular species of biliary PC.

It has been previously reported that dietary modifications affect the fatty acid composition of biliary PC (23,24,36–41). In several studies, attempts were made to find a correlation between changes in biliary PC molecular species and cholelithogenesis, but no clear cause-and-effect relationship has emerged (11–14,18,19,22,37). We used our hamster model to examine systematically how changes in dietary fatty acid composition affect the biliary PC species and whether such changes are correlated with gallstone formation. To our knowledge, this is the first study of biliary PC composition in the hamster.

In the present study, female hamsters were fed an LD supplemented with either butterfat, safflower oil, or an unsaturated fatty acid (linoleic acid, 18:2 or oleic acid, 18:1). Safflower oil and 18:2 reduced the gallstone incidence; 18:1 totally inhibited it during the 6-wk feeding period. We previously showed that dietary olive oil (71% 18:1 content) effectively lowered the induced cholelithiasis (6,7). Under the conditions employed, it does not seem to matter whether the unsaturated fatty acids are supplied in the free form or as triacylglycerols. Interestingly, additional butterfat reduced stone incidence in this model. This finding may be ascribed to the fact that butterfat contains both saturated (66%) and unsaturated (34%) fatty acids. Oleic acid (18:1) accounts for 29% of the total fatty acids in butterfat. Thus the relative proportions of the many different fatty acids in butterfat (Table 1) may play a role in gallstone development. Perhaps, some critical threshold amount of one of the unsaturated acids is important, but additional studies are needed to verify this point. It was also shown in a different hamster model that saturated fatty acids enhanced while unsaturated (18:1) reduced cholelithogenesis (42). In the present investigation, the cholesterol saturation of bile was high in all five groups studied, but was just below unity in the groups receiving safflower oil or linoleic acid. The molar cholesterol content of the biles does not provide an adequate explanation for our findings.

PC comprise more than 90% of biliary phospholipids (43,44). The four major PC species (85–88%) in hamster bile were 16:0-18:2, 16:0-18:1, 18:0-18:2, and 16:0-20:4. These PC are identical to those found in human bile (17). The predominant PC species in most mammalian biles are 16:0-18:2 and 16:0-18:1 (18,44–46). The PC transfer protein is highly specific for these two PC species (47). The addition of unsaturated fatty acids to the standard hamster diet altered the relative proportion of the biliary PC species. With added 18:2 or safflower oil, the proportion of 16:0-18:2 in PC increased by 16–18% in comparison with the control LD. When the diet was enriched with 18:1, the 16:0-18:1 species increased by 25%; when additional butterfat was added, this increase amounted to 9%. Berr *et al.* (24) fed diets rich in safflower oil, coconut oil, or fish oil to hamsters and found that the total fatty acid pattern of the bile was modified as a function of the major dietary fatty acids; however, they did not analyze the individual PC species. Kajiyama *et al.* (22) fed Dam-type diets to female hamsters to induce cholelithiasis. In their model, total biliary 18:2 decreased and 18:1 increased as stone incidence increased. However, the experimental diet was deficient in the essential fatty acid (18:2), in contrast to our diet, which contains 2% corn oil to prevent essential fatty acid deficiency.

Increased polyunsaturated fatty acid intakes reduce the conversion of linoleic acid to arachidonic acid (20:4) by limiting Δ6-desaturase activity (48). This may be the case in the present experiment, since feeding 18:2 in the form of safflower oil or as the free acid resulted in the accumulation of 18:2 in biliary PC while 20:4 remained relatively constant (Table 5). Glenn *et al.* (23) fed hamsters either a butterfat diet or a diet containing a margarine high in 18:2 and found that the proportion of 18:2 in biliary phospholipids increased in the margarine group; 20:4 also remained constant. This suggested that dietary 18:2 was not converted to 20:4 but was incorporated directly into PC.

Data on the role of specific PC species in gallstone formation have led to conflicting conclusions. For example, LaMorte *et al.* (40) reported that in prairie dogs, diets rich in linoleic acid accelerated cholesterol gallstone formation by stimulating the secretion of biliary cholesterol. In contrast to our hamster model, diets rich in linoleic acid inhibited cholesterol gallstone formation. Nevertheless, there is some evidence that the solubilization of cholesterol in bile is influenced by the nature of the fatty acids and the headgroups of the biliary phospholipids (49). It seems likely that cholesterol nucleation occurs from vesicles with a high cholesterol-to-phospholipid ratio (50–53). Nucleation time studies with model biles suggest that the cholesterol-holding capacity of vesicles depends on the hydrophobicity of the vesicular lecithin. Vesicles containing lecithins with unsaturated fatty acyl chains tend to release cholesterol more rapidly than those containing more saturated lecithins which bind cholesterol more firmly (54). The degree of acyl chain unsaturation of PC affects the molecular packing of cholesterol–lecithin monolayers (55). We have found that vesicles were present in all groups (1 to 5); conceivably the 16:0-18:1 and 16:0-18:2 lecithin species stabilize these vesicles. In model and human biles, the more saturated PC species such as 16:0-18:1 are preferentially associated with vesicles (56,57). Further studies are needed to determine whether the 16:0-18:2 and 16:0-18:1 species (which are diet-dependent in our model) favor either micelles or vesicles. Another study with model bile suggested that the more saturated lecithins decrease the initial rate of crystallization of cholesterol, possibly due to high affinity binding of such lecithins to cholesterol crystals and to each other (58). This binding could interfere with crystal nucleation since cholesterol crystallizes as filamentous crystals that are covered with a surface layer of lecithin molecules (58). An increase in either 16:0-18:1 or 16:0-18:2 could have this effect.

In conclusion, in the hormone-treated female hamster model of cholesterol cholelithiasis, supplementation of the diet with oleic or linoleic acid resulted in the incorporation of these fatty acids into biliary PC. This was associated with a decrease in the incidence of cholesterol gallstones. Diet-induced changes in biliary lecithin species may play a role in stabilizing cholesterol–phospholipid vesicles and preventing cholelithiasis.

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Platelet and Aorta Arachidonic and Eicosapentaenoic Acid Levels and *in vitro* Eicosanoid Production in Rats Fed High-Fat Diets

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ABSTRACT: There is a significant interest in the interrelationship between long-chain n-3 and n-6 fatty acids due to their ability to modulate eicosanoid production. In general, the intake of arachidonic acid (AA) results in enhanced eicosanoid production, whereas n-3 polyunsaturated fatty acids (PUFA) decrease the production of eicosanoids from AA. The purpose of this study was to investigate whether the effects of dietary AA on eicosanoid production in the rat were correlated with the AA and EPA levels in platelets and aorta (eicosanoid-producing tissues). Four groups of male Sprague-Dawley rats were fed a high-fat diet enriched with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (approximately 100 mg/day of EPA + DHA) for 24 d. During the last 10 d, the four groups were orally supplemented with 0, 30, 60, and 90 mg/day of ethyl arachidonate. A further group of rats was fed a control diet (without long-chain n-3 PUFA) for 24 d. *In vitro* aorta prostacyclin (PGI_2) production, serum thromboxane A_2 (TxA_2) production and plasma, and platelet and aorta phospholipid (PL) fatty acids were measured. Enriching the diet with n-3 PUFA resulted in significant reductions in tissue AA levels and an increase in the n-3 PUFA, particularly EPA. On this diet, the AA to EPA ratio was 1:1 in platelet PL, and it was 2:1 in the aorta PL. There were significant decreases in the *in vitro* PGI_2 and TxA_2 production compared with the control animals. The inclusion of AA in the diet resulted in marked increases in AA levels in the platelet and aorta PL with corresponding decreases in EPA. The lowest dose of AA (30 mg/rat) reversed the effects of 100 mg/day of n-3 PUFA on AA levels in platelet and aortic PL and on *in vitro* aorta PGI_2 and serum TxA_2 production. The dietary AA caused a differential (twofold) increase in TxA_2 relative to PGI_2 for all three levels of AA supplementation. There were greater changes in the levels of AA and/or EPA in platelet PL compared with the aorta PL, which might have accounted for the differential effects of these PUFA on thromboxane production compared with PGI_2 production in this study.

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It is now well established that n-3 polyunsaturated fatty acids (PUFA) can modulate the production of eicosanoids from arachidonic acid (AA). Some studies in humans have reported that these PUFA selectively decrease thromboxane (TxA_2) production while exerting only a modest suppressive effect on prostacyclin (PGI_2) synthesis (1,2). In other studies in rats and mice, the production of both of these eicosanoids was decreased by n-3 PUFA (3–6).

Although most tissue AA is derived from linoleic acid, there is interest in the consequences of the ingestion of AA as it has been argued that this could lead to an overproduction of eicosanoids, particularly TXA_2 and, therefore, an increase in thrombosis (7). Several animal studies have shown that dietary AA increases the production of eicosanoids (TxA_2 and PGI_2) and leukotrienes (8–10).

Dietary AA is found in foods of animal origin and is usually present together with long-chain n-3 PUFA (11,12). We have proposed that the n-3 PUFA present in diets containing animal products would differentially decrease TxA_2 production compared with PGI_2 production (11). However, Li *et al.* (10) have reported that dietary AA, when present at the same level in the diet as the EPA, abolishes the depressive effect of dietary eicosapentaenoic acid (EPA) on *in vitro* eicosanoid production in mice. These authors showed that when AA and EPA were present in the diet in equal quantities, there was very little EPA incorporated into the liver phospholipids (PL).

The purpose of this study was to investigate whether the effects of dietary AA on eicosanoid production in the rat were correlated with the AA and EPA levels in platelets and aorta (eicosanoid-producing tissues). The dietary protocol used was one which we have shown is capable of responding to dietary AA by increases in eicosanoid production (9). In this model, rats are fed a high saturated fat diet for about two weeks, which reduces the tissue AA and eicosanoid levels (13). Addition of AA to this dietary regime results in significant rises in tissue AA and eicosanoid levels (9). Using this scheme, we proposed to add 100 mg/day of long-chain n-3 PUFA to the saturated fat diet to further depress eicosanoid synthesis and to test the effect of the addition of 30, 60, or 90 mg of AA on platelet and aorta fatty acid levels and eicosanoid production.

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; RIA, radioimmunoassay; PGI_2, prostacyclin; PL, phospholipid; PUFA, polyunsaturated fatty acid; Tx, thromboxane.

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MATERIALS AND METHODS

Materials. Ethyl arachidonate (>99% pure) was purchased from Nu-Chek-Prep (Elysian, MN). Ethyl arachidonate (91% AA, 9% γ-linolenic acid) was a generous gift from Suntory Ltd., Institute for Biomedical Research (Osaka, Japan). Sodium pentothal was purchased from Abbott Australasia (Sydney, Australia). Scintillation proximity assay radioimmunoassay (RIA) kits for 6-keto-$PGF_{1\alpha}$ and thromboxane B_2 (TxB_2) were purchased from Amersham Australia Pty. Ltd. (Sydney, Australia). All solvents used in lipid extractions were of nanograde standard supplied by Mallinckrodt Australia Pty. Ltd. (Melbourne, Australia). The dry n-3 encapsulated fish oil powder was a generous gift from Danochemo A/S, Malmparken (Ballerup, Denmark). Sodium metrizoate was purchased from Nycomed (Oslo, Norway). The capillary column was supplied by Restek Corporation (Chromalytic Technology Pty. Ltd., Melbourne, Australia).

Animals. Forty male Sprague-Dawley rats weighing between 80–130 g were housed in a temperature controlled (22 ± 1°C) room with a 12-h light–dark cycle. The animals were randomly assigned to five dietary groups of eight animals each; one group was fed the control diet for 24 d while the other four groups were fed the control diet plus n-3 PUFA for the 24 d, with variable amounts of AA being provided for the final 10 d. Food and water were provided *ad libitum,* and body weights and food intakes were recorded every day. The animals were not fasted overnight prior to sacrifice.

Diets. The nutrient composition of the experimental diets is shown in Table 1, and the fatty acid compositions of the semisynthetic diets are given in Table 2. Two diets were prepared, a control diet and another diet enriched with n-3 fatty acids, providing a combined total of 100 mg of EPA and docosahexaenoic acid (DHA) per 15 g of food. The diets were designed to provide 50% of the energy as fat, 30% as carbohydrate and 20% as protein, and were based on those used in our previous studies (9,13–15). Vitamins and minerals were added to the diets in accordance with the recommendations by the American Institute of Nutrition (16). The n-3 PUFA were incorporated into the diet as a dry n-3 powder which consists of fish oil encapsulated in a gelatine coating. In this form, oxidation of the fish oils is likely to be minimal. The main fatty acids of the encapsulated fish oil were 14:0 (7.3%), 16:0 (14.9%), 16:1 (8.7%), 20:5n-3 (17.1%), 22:5n-3 (2.3%), and 22:6n-3 (11.2%). Gelatine was added to the control diet to balance that present in the dry n-3 powder. During the last 10 d of the study, four groups of animals receiving the n-3 enriched diet were orally supplemented with 30, 60, and 90 mg/day of ethyl arachidonate. The ethyl arachidonate was given orally to minimize oxidation. Four animals in each of the AA-supplemented group received ethyl AA from Nu-Chek-Prep and the other four received the Suntory ethyl AA.

. In vitro *PGI_2 production.* At the conclusion of the 24-d feeding period, the animals were anesthetized intraperitoneally with sodium pentothal (100 mg/kg body weight). Blood was taken by cardiac puncture for TxB_2 production and plasma fatty acid analysis. Immediately after blood collec-

TABLE 1
Composition of the Experimental Diets

Constituents (g/100/g)	Control	n-3 PUFA enriched[a]
Casein	22.1	22.4
Corn starch	36.7	37.0
Butter	27.5	25.0
Dry n-3 powder	—	9.4
Gelatine	7.0	—
Mineral mix[b]	5.0	4.6
Vitamin mix[b]	1.4	1.3
Choline chloride[b]	0.3	0.3
Methionine[b]	0.4	0.4
Composition (% energy)		
Protein	20	20
Carbohydrate	30	30
Fat	50	50

[a]The n-3 polyunsaturated fatty acid (PUFA)-enriched diet refers to a diet containing butter which was enriched with eicosapentaenoic acid and docosahexaenoic acid.
[b]These values vary between diets because they are calculated as a percentage of energy density to give a constant amount per gram of dry weight of diet (that is, butter contains 20% water and in the latter diet there is a lower percentage of butter).

TABLE 2
Fatty Acid Composition (wt%) of the Experimental Diets

Fatty acids	Study diets	
	Control	n-3 PUFA-enriched[a]
6:0[b]	3.4	3.0
8:0	1.7	1.8
10:0	3.6	3.2
12:0	3.7	3.2
14:0	11.8	10.9
14:1	2.1	1.8
15:0	1.3	1.2
16:0	29.7	27.4
16:1	2.8	3.6
17:0	0.9	0.9
18:0	10.4	10.0
18:1	22.6	21.5
18:2n-6	1.4	1.4
19:0	0.5	1.0
18:3n-3	0.6	0.7
20:0	0.2	0.2
20:1	0.9	1.2
18:4n-3	—	0.6
20:4n-6	—	0.2
20:4n-3	—	0.1
20:5n-3	—	1.7
22:5n-3	—	0.3
22:6n-3	—	1.1
24:0	—	0.1
Saturated	67.2	62.9
Monoenoic	28.4	28.1
Polyenoic	2.0	6.1
n-6/n-3 Ratio	2.3	0.4

[a]The n-3 PUFA-enriched diet refers to a butter diet which was enriched with eicosapentaenoic acid and docosahexaenoic acid. See Table 1 for abbreviation.
[b]Fatty acids identified by retention time in excess of 0.1% reported.

tion, the animals were perfused with Krebs Ringer Bicarbonate buffer at pH 7.4 administered *via* the left ventricle to flush the remaining blood from the animal, leaving the artery preparation free of adhering blood thrombi. The abdominal aorta was dissected, split longitudinally, stabilized in Krebs Ringer Bicarbonate buffer at 37°C for 15 min in a water bath, and incubated for a further 15 min as described in detail previously (17). Aliquots were taken from the incubation medium and stored at –20°C for RIA determination of 6-keto $PGF_{1\alpha}$ (stable metabolite of PGI_2). It is highly unlikely that the antibody used in this RIA technique differentiates between the dienoic and trienoic metabolites (6-keto $PGF_{1\alpha}$ and Δ^{17}-6-keto $PGF_{1\alpha}$). At the end of the incubation, aorta segments were removed, blotted on filter paper, and then frozen in liquid nitrogen and weighed. The connective tissue was cleared from the aorta by cutting the top of the aorta arch. The remaining aorta was stored in Krebs Ringer Bicarbonate buffer at –20°C for later determination of the fatty acid composition.

In vitro *thromboxane production.* Blood taken by cardiac puncture was incubated for 1 h at 37°C and centrifuged for 2 min in a Beckman (Beckman Instruments, Melbourne, Australia) benchtop microfuge. The serum was removed and stored at –20°C for the RIA measurement of serum TxB_2 production (stable metabolite of TxA_2), using the method described previously by Block *et al.* (18). It is highly unlikely that the antibody used in this RIA technique differentiates between the dienoic and trienoic TxA metabolites.

Isolation of platelets. Blood taken by cardiac puncture was immediately placed into two 5-mL tubes containing 0.105 M sodium citrate. These tubes were kept between 30 and 35°C until the analyses could be completed. Platelet-rich plasma was prepared by spinning the citrate tubes at 200 × *g* for 10 min. Using a plastic pipette, the platelet-rich plasma was gently transferred into a plain 10-mL tube, then 2 mL of 13.7% sodium metrizoate (diluted from 32.8% using sodium/glucose buffer) was gently added to the bottom of the plain tube (that is, under the platelet-rich plasma layer) using a silanized glass pipette. A further 2-mL of 32.8% sodium metrizoate was gently added below the 13.7% sodium metrizoate layer using a silanized glass pipette to provide a density gradient for the collection of platelets. After centifugation (10 min) at 3000 × *g* at room temperature, the separated platelets (which were concentrated between the density gradient created by the two metrizoate layers) were transferred into a silanized glass tube containing 10 mL of tris saline buffer (0.05 M, pH 7.2). The tube was inverted several times and placed in a centrifuge set at 2000 × *g*, for 10 min. The buffer was discarded and the platelet pellet suspended in 10 mL of chloroform/methanol (1:1). The tube was vortexed and left in a freezer (–20°C) until the analysis of the fatty acids.

Fatty acid composition of plasma, aorta, and platelet PL. The lipids were extracted from plasma, thoracic aorta, and platelets by chloroform/methanol (1:1) lipid extraction, followed by thin-layer chromatography separation of the fatty acid classes to obtain the PL fraction as described previously (14). The PL were subjected to saponification and methylation (14), and the resultant fatty acid methyl esters were separated by capillary gas–liquid chromatography on a 60 m × 0.32 mm i.d. Rtx-2330 crossbonded column (phase thickness 0.25 μm). Helium was used as the carrier gas with a flow rate of 43 cm/s. Cold on-column injection was used, and the flame-ionization detector temperature was 280°C. Individual fatty acid methyl esters were identified by comparing their retention time with those of a standard fatty acid methyl ester mixture and by the inclusion of an internal standard of diheptadecanoyl phosphatidylcholine in the aliquots prior to methyl ester formation. The results were corrected by the use of response factors which were calculated using standard fatty acid methyl esters.

Statistical analysis. Differences between groups were established using analysis of variance with the aid of a multiple comparison test (Tukey-HSD) to establish where the differences between groups occurred. The *in vitro* eicosanoid data was not consistent with the hypothesis of normality. Therefore, differences between groups were tested by the Kruskal-Wallis nonparametric analysis of variance and Dunn's multiple comparison test. Statistical analyses were performed on Minitab Version 8 (Minitab Inc., State Collage, PA). All results are expressed as mean ± SEM, $P < 0.05$ for all significant changes.

RESULTS

The main source of fat in both diets was butter, and this was evident from the diet fatty acid composition (Table 2) which had a high proportion of saturated fatty acids, monounsaturated fatty acids, and low levels of linoleic acid and α-linolenic acid. The control diet enriched with n-3 PUFA was designed so that animals consuming 15 g/day of food would ingest 100 mg/day of EPA and DHA. In this diet, the fish oil provided EPA and DHA at 1.7 and 1.1% of the total dietary fatty acids, respectively, and the total n-3 fatty acid level was 4.5% compared with the control diet, which contained 2.0% n-3 PUFA.

There were no significant differences in the average weight gain or food intake between groups. The average initial weight for animals per group was 102 g, and the average final weight was between 281–304 g. The average weight gain between groups varied from 7.5 to 8.2 g/day/rat. Food intake for the animals fed the control diet and that enriched with n-3 PUFA was 15.5 g/day/rat.

Enriching the control diet with n-3 PUFA led to substantial increases in EPA and 22:5n-3, but not in DHA (except for the aortic PL) in comparison with the control rats (Tables 3, 4, and 5). The increases in the n-3 PUFA were generally at the expense of 18:1n-9, 20:3n-9, 18:2n-6, and 20:3n-6. There was a fivefold decrease in the AA to EPA ratio in the plasma, aorta, and platelets of the animals fed the n-3 PUFA-enriched diet in comparison with the control rats. In the plasma and the platelets of the animals fed the n-3 enriched PUFA diet, the AA to EPA ratio was about 1:1, although it was 2:1 in the

TABLE 3
Fatty Acid Composition of Plasma Phospholipids in Rats fed an n-3 PUFA-Enriched Diet with Varying Doses of Arachidonic Acid (wt% of total fatty acids)[a]

Fatty acid	Control ($n = 8$)	n-3 PUFA-enriched + AA			
		0 mg AA ($n = 8$)	30 mg AA ($n = 8$)	60 mg AA ($n = 8$)	90 mg AA ($n = 8$)
16:0	19.5 ± 0.3[a]	22.9 ± 0.3[b]	21.9 ± 0.3[b,c]	22.0 ± 0.2[b,c]	21.6 ± 0.2[c]
16:1	2.5 ± 0.2[a]	2.0 ± 0.1[b]	1.7 ± 0.1[b]	1.9 ± 0.1[b]	1.7 ± 0.1[b]
18:0	17.3 ± 0.9	16.2 ± 0.8	16.6 ± 0.7	15.9 ± 0.7	17.6 ± 0.6
18:1n-9	19.3 ± 0.5[a]	14.7 ± 0.3[b]	12.8 ± 0.1[c]	12.4 ± 0.3[c]	12.1 ± 0.3[c]
18:2n-6	13.7 ± 0.3[a]	9.9 ± 0.1[b]	8.1 ± 0.2[c]	7.7 ± 0.2[c,d]	7.2 ± 0.3[d]
20:3n-9	1.8 ± 0.1[a]	0.3 ± 0.0[b]	0.1 ± 0.0[c]	0.0 ± 0.0[c]	0.0 ± 0.0[c]
20:3n-6	1.9 ± 0.0[a]	1.1 ± 0.0[b]	0.8 ± 0.0[c]	0.7 ± 0.0[c,d]	0.7 ± 0.0[d]
20:4n-6	8.6 ± 0.5[a]	8.6 ± 0.3[a]	17.9 ± 0.4[b]	21.2 ± 0.7[c]	23.0 ± 0.8[c]
20:5n-3	1.8 ± 0.2[a]	10.2 ± 0.5[b]	8.4 ± 0.4[c]	7.0 ± 0.4[c,d]	6.1 ± 0.4[d]
22:4n-6	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
22:5n-6	0.2 ± 0.0[a]	0.1 ± 0.0[b]	0.1 ± 0.0[b]	0.1 ± 0.0[b]	0.0 ± 0.0[c]
22:5n-3	0.7 ± 0.0[a]	2.6 ± 0.1[b]	2.0 ± 0.1[c]	2.0 ± 0.1[c]	1.7 ± 0.1[d]
22:6n-3	8.0 ± 0.4[a]	7.3 ± 0.3[a]	6.2 ± 0.5[b]	5.5 ± 0.3[b,c]	4.6 ± 0.1[c]
n-6 Total	24.1 ± 0.6[a]	19.8 ± 0.4[b]	27.1 ± 0.5[c]	29.9 ± 0.7[d]	31.1 ± 0.6[d]
n-3 Total	10.5 ± 0.3[a]	20.1 ± 0.6[b]	15.9 ± 0.6[c]	14.1 ± 0.8[c]	11.7 ± 0.6[a,d]
n-6/n-3	2.4 ± 0.1[a]	1.0 ± 0.0[b]	1.7 ± 0.1[c]	2.2 ± 0.1[a]	2.7 ± 0.1[d]
AA/EPA	5.0 ± 0.3[a]	0.9 ± 0.0[b]	2.1 ± 0.1[c]	3.0 ± 0.2[d]	3.8 ± 0.3[d]

[a]Rats were fed the n-3 PUFA-enriched diet which contained 60 mg eicosapentaenoic acid (EPA)/day and 40 mg docosahexaenoic acid/day. These animals were supplemented with ethyl arachidonate (0, 30, 60, or 90 mg per day) during the last ten days of the study. AA, arachidonic acid; see Table 1 for other abbreviation. Results are shown as mean ± SEM. Fatty acids identified by retention time in excess of 0.1% reported. The values in the same column with different lettered superscripts (a, b, c, d) differ significantly, (analysis of variance, $P < 0.05$). Rows of data with no following letters indicate no significant differences.

TABLE 4
Fatty Acid Composition of Platelet Phospholipids in Rats Fed an n-3 PUFA-Enriched Diet with Varying Doses of Arachidonic Acid (wt% of total fatty acids)[a]

Fatty acid	Control ($n = 8$)	n-3 PUFA-enriched + AA			
		0 mg AA ($n = 8$)	30 mg AA ($n = 8$)	60 mg AA ($n = 8$)	90 mg AA ($n = 8$)
16:0	26.2 ± 0.5	27 ± 0.9	27.4 ± 1.0	28.1 ± 0.3	27.4 ± 0.4
16:1	2.1 ± 0.3[a]	2.1 ± 0.2[a]	0.8 ± 0.2[b]	1.3 ± 0.3	1.5 ± 0.2
18:0	12.4 ± 0.6	12.3 ± 0.5	13.5 ± 0.6	12.7 ± 0.4	12.2 ± 0.5
18:1n-9	12.9 ± 0.5[a]	10.5 ± 0.3[b]	9.7 ± 0.2[b]	9.0 ± 0.3[c]	8.3 ± 0.2[c]
18:2n-6	5.9 ± 0.3[a]	5.0 ± 0.2[b]	3.9 ± 0.2[c]	3.1 ± 0.2[c,d]	2.9 ± 0.1[d]
20:3n-9	2.0 ± 0.2[a]	0.5 ± 0.1[b]	0.3 ± 0.1[b]	0.2 ± 0.0[b]	0.1 ± 0.0[c]
20:3n-6	1.9 ± 0.1[a]	0.9 ± 0.1[b]	0.7 ± 0.0[b]	0.6 ± 0.0[c]	0.6 ± 0.0[c]
20:4n-6	19.0 ± 1.0[a]	12.1 ± 0.5[b]	21.2 ± 0.3[a,c]	24.0 ± 0.6[c]	26.7 ± 0.6[d]
20:5n-3	3.8 ± 0.2[a]	12.3 ± 0.6[b]	6.8 ± 0.4[c]	6.4 ± 0.3[c]	5.5 ± 0.3[c]
22:4n-6	1.0 ± 0.1[a]	0.7 ± 0.0[a]	1.6 ± 0.1[b]	1.9 ± 0.1[b,c]	2.2 ± 0.1[c]
22:5n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
22:5n-3	2.0 ± 0.1[a]	5.0 ± 0.2[b]	4.2 ± 0.2[c]	4.1 ± 0.12[c]	3.8 ± 0.1[c]
22:6n-3	1.7 ± 0.1[a]	1.8 ± 0.1[a]	1.5 ± 0.1[b]	1.2 ± 0.1[b,c]	1.1 ± 0.0[c]
n-6 Total	28.0 ± 1.0[a]	18.8 ± 0.6[b]	27.5 ± 0.2[a]	29.8 ± 0.5[a]	32.6 ± 0.5[c]
n-3 Total	7.5 ± 0.3[a]	19.1 ± 0.8[b]	12.4 ± 0.7[c]	11.7 ± 0.3[c]	10.5 ± 0.5[c]
n-6/n-3	3.8 ± 0.2[a]	1.0 ± 0.0[b]	2.2 ± 0.1[c]	2.6 ± 0.1[c]	3.1 ± 0.1[d]
AA/EPA	5.1 ± 0.4[a]	1.0 ± 0.1[b]	3.2 ± 0.1[c]	3.8 ± 0.2[c]	4.9 ± 0.3[a]

[a]Rats were fed the n-3 PUFA-enriched diet which contained 60 mg eicosapentaenoic acid (EPA)/day and 40 mg docosahexaenoic acid/day. These animals were supplemented with ethyl arachidonate (0, 30, 60, or 90 mg per day) during the last ten days of the study. AA, arachidonic acid; see Tables 1 and 3 for abbreviations. Results are shown as mean ± SEM. Fatty acids identified by retention time in excess of 0.1% reported. The values in the same column with different lettered superscripts (a, b, c, d) differ significantly, (analysis of variance, $P < 0.05$). Rows of data with no following letters indicate no significant differences.

TABLE 5
Fatty Acid Composition of Aorta Phospholipids in Rats Fed an n-3 PUFA-Enriched Diet with Varying Doses of Arachidonic Acid (wt% of total fatty acids)[a]

Fatty acid	Control ($n = 8$)	n-3 PUFA-enriched + AA			
		0 mg AA ($n = 8$)	30 mg AA ($n = 8$)	60 mg AA ($n = 8$)	90 mg AA ($n = 8$)
16:0	20.3 ± 0.3[a]	21.7 ± 0.2[b]	22.0 ± 0.4[b]	21.1 ± 0.4	21.1 ± 0.3
16:1	2.4 ± 0.2	2.1 ± 0.2	1.9 ± 0.2	2.3 ± 0.2	2.0 ± 0.1
18:0	14.2 ± 0.6	14.7 ± 0.5	14.9 ± 1.0	14.7 ± 0.6	15.1 ± 0.7
18:1n-9	17.0 ± 0.5[a]	16.1 ± 0.3[a,b]	15.1 ± 0.3[b]	14.8 ± 0.4[b]	14.8 ± 0.3[c]
18:2n-6	5.2 ± 0.2[a]	4.2 ± 0.1[b]	3.4 ± 0.1[c]	3.2 ± 0.1[c]	3.1 ± 0.1[c]
20:3n-9	2.2 ± 0.1[a]	1.1 ± 0.1[b]	1.0 ± 0.1[b]	0.9 ± 0.1[b]	0.8 ± 0.0[c]
20:3n-6	1.8 ± 0.0[a]	1.3 ± 0.0[b]	1.1 ± 0.0[c]	1.0 ± 0.0[c]	1.1 ± 0.1[c]
20:4n-6	18.0 ± 0.5[a]	13.2 ± 0.3[b]	18.3 ± 0.3[b]	19.8 ± 0.7[a,c]	20.9 ± 0.5[c]
20:5n-3	1.7 ± 0.1[a]	6.2 ± 0.3[b]	4.3 ± 0.3[c]	3.7 ± 0.2[c,d]	3.2 ± 0.2[d]
22:4n-6	1.6 ± 0.0[a]	0.9 ± 0.0[b]	1.2 ± 0.0[c]	1.3 ± 0.0[c]	1.5 ± 0.0[d]
22:5n-6	0.5 ± 0.0[a]	0.3 ± 0.0[b]	0.3 ± 0.0[b]	0.3 ± 0.0[b]	0.3 ± 0.0[b]
22:5n-3	2.0 ± 0.1	4.8 ± 0.0[b]	4.3 ± 0.1[c]	4.3 ± 0.1[c]	4.1 ± 0.1[c]
22:6n-3	4.7 ± 0.2[a]	6.5 ± 0.2[b]	6.2 ± 0.2[b,c]	6.0 ± 0.2[b,c]	5.6 ± 0.2[c]
n-6 Total	27.1 ± 0.6[a]	19.9 ± 0.3[b]	24.3 ± 0.6[c]	25.6 ± 0.7[a]	26.8 ± 0.4[a]
n-3 Total	8.4 ± 0.3[a]	17.4 ± 0.4[b]	14.8 ± 0.6[c]	14.0 ± 0.4[c]	12.9 ± 0.3[d]
n-6/n-3	3.2 ± 0.1[a]	1.1 ± 0.0[b]	1.7 ± 0.1[c]	1.8 ± 0.0[c]	2.1 ± 0.0[d]
AA/EPA	11.1 ± 0.7[a]	2.2 ± 0.1[b]	4.4 ± 0.3[c]	5.3 ± 0.2[c]	6.7 ± 0.2[d]

[a]Rats were fed the n-3 PUFA-enriched diet which contained 60 mg eicosapentaenoic acid (EPA)/day and 40 mg docosahexaenoic acid/day. These animals were supplemented with ethyl arachidonate (0, 30, 60, or 90 mg per day) during the last ten days of the study. AA, arachidonic acid; see Tables 1 and 3 for abbreviations. Results are shown as mean ± SEM. Fatty acids identified by retention time in excess of 0.1% reported. The values in the same column with different lettered superscripts (a, b, c, d) differ significantly (analysis of variance, $P < 0.05$). Rows of data with no following letters indicate no significant differences.

aorta. There were substantial reductions in the *in vitro* production of immunoreactive PGI_2 (24% reduction) and TxA_2 (54% reduction) in the rats fed the n-3 PUFA-enriched diet compared with the control animals (Figs. 1 and 2, respectively). While the ratio of TxA to PGI declined by almost 60%, this was not significant (Fig. 3).

The inclusion of AA to the diet of rats fed the n-3 PUFA diet resulted in dose-dependent increases in AA levels in plasma, aorta, and platelet PL. Two different ethyl arachidonate preparations were used for this study. One was supplied by Nu-Chek-Prep, and the other was from Suntory Ltd. For each group of animals supplemented with AA, four of the eight animals received one preparation and the remaining four animals the other. Unpaired Student's *t*-tests (results not supplied) showed that there were no significant differences in the AA levels of the plasma, platelet, aorta PL, or *in vitro* PGI_2 and TxA_2 production within dietary groups regardless of which type of AA was administered. Therefore, the results of the two groups of four animals were combined and discussed as one group consisting of eight animals.

There was a greater increase (more than a doubling) of AA levels for plasma and platelet PL compared with the aortic PL in the rats supplemented with 90 mg/day, while the EPA levels in all tissues fell to about half the level of the 0 mg/day AA group (fed the n-3 PUFA-enriched diet). The 30 mg/day dose of AA resulted in significant increases (more than a doubling) in the AA to EPA ratio all three tissues. AA supplementation resulted in significant reductions in EPA, 22:5n-3, and DHA in all three tissues.

The inclusion of AA in the diet of rats receiving the n-3 PUFA-enriched diet resulted in significant increases for both vascular PGI_2 and serum TxA. The TxA and PGI production in the rats supplemented with 30 mg of AA/day was signifi-

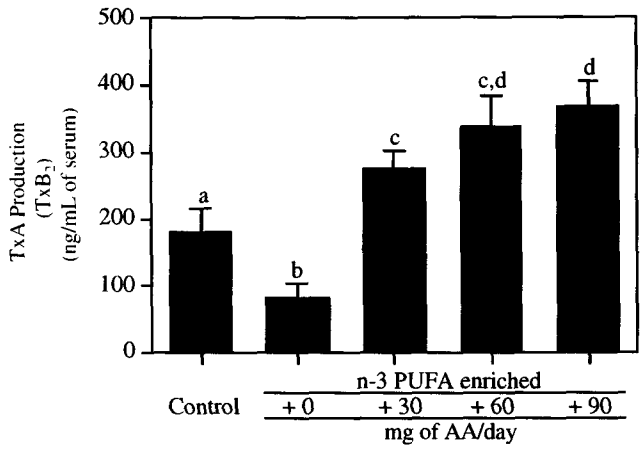


FIG. 1. *In vitro* serum thromboxane A_2 (TxA_2) production measured as TXB_2 (ng/mL serum); mean ± SEM. The rats fed the n-3 polyunsaturated fatty acid (PUFA) enriched diet were orally supplemented with ethyl arachidonate (0, 30, 60, and 90 mg per day) during the last ten days of the study. The columns with different lettered superscripts (a, b, c, d) differ significantly (analysis of variance, $P < 0.05$). AA, arachidonic acid.

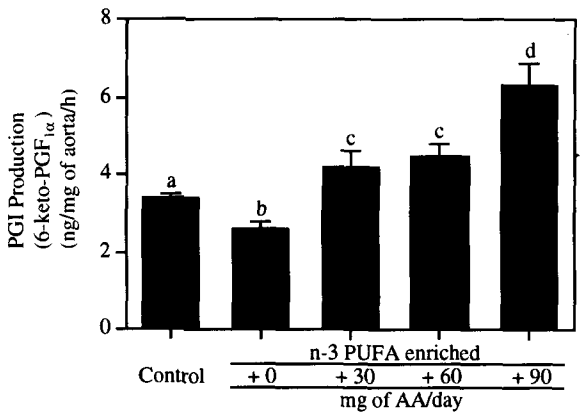


FIG. 2. *In vitro* aorta prostacyclin (PGI_2) production measured as 6-keto-$PGF_{1\alpha}$ (ng/mg aorta/h) mean ± SEM. The rats fed the n-3 PUFA-enriched diet were orally supplemented with ethyl arachidonate (0, 30, 60, and 90 mg per day) during the last ten days of the study. The columns with different lettered superscripts (a, b, c, d) differ significantly (analysis of variance, $P < 0.05$). See Figure 1 for abbreviations.

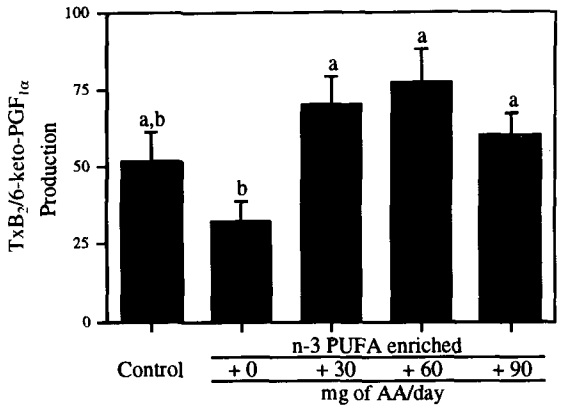


FIG. 3. Ratio of *in vitro* serum thromboxane (TXA_2) production measured as TXB_2 (ng/mL serum) relative to *in vitro* aorta prostacyclin (PGI_2) production measured as 6-keto-$PGF_{1\alpha}$ (ng/mg aorta/hour); mean ± SEM. The rats fed the n-3 PUFA-enriched diet were orally supplemented with ethyl arachidonate (0, 30, 60, and 90 mg per day) during the last ten days of the study. The columns with different lettered superscripts (a, b, c, d) differ significantly (analysis of variance, $P < 0.05$). See Figure 1 for abbreviations.

cantly greater than the 0 mg/day AA group (who were fed 100 mg/day of long-chain n-3 PUFA). The ratio of TxA to PGI was more than doubled by the inclusion of AA in the diet (Fig. 3).

DISCUSSION

The purpose of this study was to investigate whether the effects of dietary AA on eicosanoid production in the rat were correlated with the AA and EPA levels in platelets and aorta (eicosanoid-producing tissues). In order to do this, we studied the consequences of combined dietary intakes of AA and long-chain n-3 PUFA on platelet and aorta fatty acids and eicosanoid production in the rat.

When the rats were fed the diet containing 100 mg/day of EPA plus DHA for 24 d, there were differential effects on the AA to EPA ratios in platelets and aorta PL. The AA/EPA ratio was 1:1 in the platelet PL after the n-3 PUFA period, compared with 2:1 in the aorta PL. The n-3 PUFA-enriched diet was associated with significant reductions in the *in vitro* production of immunoreactive TxA_2 (54%) and PGI_2 (24%) in the group fed the n-3 PUFA, as compared with the control animals. A possible explanation for the greater reduction in TxA_2 production might relate to the lower AA/EPA ratio in the platelets.

The second finding of these experiments was that increasing the level of AA supplementation in rats fed the diet enriched with n-3 PUFA led to significant increases in AA levels in the plasma, platelets, and aorta PL at the expense of the n-3 PUFA, particularly EPA (Tables 3, 4, and 5). The lowest dose of AA (30 mg/rat) reversed the effects of 100 mg/rat of EPA plus DHA on both platelet and aorta PL, by restoring the AA levels to that equal to the control rats. These fatty acid changes were associated with significant increases for both *in vitro* aorta PGI_2 and serum TxA_2 production. This extends the observations reported by Li *et al.* (10) in mice who showed that equal amounts of AA and EPA overcame the inhibitory effects of EPA on *in vitro* eicosanoid production. In our experiment, only 30 mg AA/day was required to prevent the inhibitory effect of 100 mg/day of n-3 PUFA.

The third finding of this study was that, in the rats fed both AA and n-3 PUFA for ten days, there was a differential (twofold) increase in TxA production relative to PGI production for all three levels of AA supplementation (Fig. 3). A possible explanation could be attributed to the substantially increased availability of AA in platelets compared with aorta, following AA supplementation (the proportion of AA in the platelet PL increased from 12.1 to 26.7% compared with the aorta PL, where the AA level increased from 13.3 to 20.9%). In these studies, we are aware that the RIA procedures used are unlikely to distinguish between the diene and triene forms of the Tx and PGI; however, we do not believe this would substantially alter the conclusions drawn in the AA supplementation period of the study.

We have noted previously (17) that *in vitro* PGI_2 production is dependent on the level of AA in the aorta PL, and *in vitro* TxA_2 production is related to the level of AA in the platelet PL. The results of the present study support these observations, with the platelet AA levels being significantly correlated with *in vitro* TxA_2 production irrespective of the dietary regimen ($r = +0.713$, $P < 0.05$). Likewise, aortic AA levels and PGI_2 were also similarly correlated ($r = +0.559$, $P < 0.05$). Based on these *in vitro* data, this study highlights the importance of AA levels in eicosanoid-producing tissues compared with that of the n-3 PUFA.

In conclusion, these results show that 30 mg/day of dietary AA abolishes the depression of Tx and PGI production by 100 mg/day of n-3 PUFA. The probable explanation for these results was that the 30 mg/day of dietary AA restored the AA levels in platelet and aortic PL to a level equal or greater than

that of the control animals. These data imply that AA has a greater affinity for esterification into platelet and aorta PL than the long-chain n-3 PUFA. The greater magnitude of the changes in the levels of AA and/or EPA in platelet PL compared with the aorta PL might have accounted for the differential effects of these PUFA on Tx production compared with PGI production in this study.

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The Ratio of n-6 to n-3 Polyunsaturated Fatty Acids in the Rat Diet Alters Serum Lipid Levels and Lymphocyte Functions

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ABSTRACT: Previous studies have reported that feeding rats diets rich in fish oils, which contain high proportions of the n-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic and docosahexaenoic acids, results in lowering of blood lipid levels and suppression of lymphocyte functions tested *ex vivo* and *in vivo*. The effects of other n-3 PUFA, such as α-linolenic acid, which is found in high proportions in linseed oil, are not as well documented. Therefore, in the present study, weanling male rats were fed for six weeks on one of five high-fat (20% by weight) diets made by mixing together sunflower and linseed oils; the resulting blends had n-6/n-3 PUFA ratios of 112.5:1 (pure sunflower oil), 14.8:1, 6.5:1, 0.8:1, and 0.33:1 (pure linseed oil); the levels of all other components in the diet were identical. The final body weight and total dissectable fat were lowest in rats fed the pure linseed oil diet. Serum cholesterol, triacylglycerol and nonesterified fatty acid concentrations decreased as the n-6/n-3 PUFA ratio of the diet decreased. The fatty acid composition of the serum and of spleen lymphocytes was influenced by the diet fed—there was a progressive decrease in the proportions of linoleic and arachidonic acids and a progressive increase in the proportion of α-linolenic acid as the n-6/n-3 PUFA ratio of the diet decreased. Eicosapentaenoic and docosahexaenoic acids were detected in the serum but not in spleen lymphocytes. Inclusion of α-linolenic acid in the diet resulted in significant suppression of spleen lymphocyte proliferation in response to the T-cell mitogen concanavalin A and in spleen lymphocyte natural killer cell activity, both measured *ex vivo*. The localized graft vs. host response, a measure of cell-mediated immunity *in vivo*, progressively decreased as the n-6/n-3 PUFA ratio of the diet decreased. Thus, this study shows that dietary α-linolenic acid results in lowered blood lipid levels and suppressed lymphocyte functions *ex vivo* and *in vivo*. With respect to these effects, α-linolenic acid is as potent as dietary fish oil.

Lipids 31, 737–745 (1996).

Fat constitutes a large proportion of the Western diet, accounting for approximately 35 to 40% of energy intake. For some time there has been evidence that both the amount and type of dietary fat play a role in the health of the individual. For example, the consumption of lipids which contain a high proportion of saturated fatty acids has been linked with an increased risk of developing coronary heart disease (CHD), and this may be caused partly by the elevated blood cholesterol and triacylglycerol (TAG) concentrations which result from the consumption of such diets (1). Hence, there has been much interest in the use of diets containing unsaturated, rather than saturated, fatty acids to prevent the development of CHD. Consumption of fish oils, which are rich in the n-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic and docosahexaenoic (20:5n-3 and 22:6n-3, respectively), has been reported to result in significant lowering of blood lipid levels in both humans (see Ref. 2 for a review) and in rats (3–8). Therefore, as might be expected from the link between elevated blood lipid concentrations and development of CHD, fish oil consumption is associated with lowered mortality from CHD (9–12).

In addition to a potential effect upon blood lipid levels and CHD, dietary lipids may have immunomodulatory effects. An immunosuppressive effect of dietary fish oils is suggested by epidemiological studies which show that populations such as Greenland Eskimos, who consume large quantities of fish oils, have a very low incidence of inflammatory and autoimmune disorders (12), and by clinical studies which show that fish oil supplementation has some beneficial effects in rheumatoid arthritis, multiple sclerosis, and psoriasis, and also prolongs the survival of grafts (see Ref. 13 references therein). The potential clinical use of fish oils has given rise to a number of studies investigating the effects of fatty acids and dietary oils upon immune cell functions. *In vitro* studies have shown that n-3 PUFA, or TAG containing n-3 PUFA, inhibit a number of lymphocyte functions, including proliferation (14–21), interleukin-2 production (17,18,20), activation marker expression (17), natural killer (NK) cell activity (22), and antigen presentation (23). These studies indicate that consumption of n-3 PUFA in the diet might suppress lymphocyte functions and, thus, the immune response.

Studies of the effects of dietary lipids upon lymphocyte functions have been largely restricted to investigating lymphocyte proliferation in response to various stimuli. A num-

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Abbreviations: CHD, coronary heart disease; Con A, concanavalin A; LDH, lactate dehydrogenase; LT, leukotriene; NEFA, nonesterified fatty acid; NK, natural killer; PBS, phosphate-buffered saline; PLN, popliteal lymph node; PUFA, polyunsaturated fatty acids, TAG, triacylglycerol.

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ber of studies have shown that feeding laboratory animals (rats, mice, rabbits, chickens) diets rich in n-3 PUFA [canola, linseed, fish oils; the former two oils contain α-linolenic acid (18:3n-3)] results in suppressed lymphocyte proliferation *ex vivo* (13,24–30); investigations which have directly compared the effects of these oils indicate that fish oil is more suppressive than linseed oil which is, in turn, more suppressive than canola oil (see Refs. 31, 32 and references therein). Diets rich in n-6 PUFA appear to be less suppressive than those containing n-3 PUFA or even to be without effect (see Refs. 13,31,32 and references therein). *Ex vivo* cytotoxic T lymphocyte activity is also suppressed by feeding laboratory animals fish, linseed, and n-6 PUFA-containing oils (33–37); again, fish oil is more suppressive than linseed oil, which is more suppressive than n-6 PUFA-containing oils. Recent studies have reported that feeding rats or mice fish oil results in suppressed NK cell activity compared with feeding linseed or n-6 PUFA-containing oils (29,36,38–40). Taken together, these *ex vivo* observations indicate that consumption of diets containing n-3 PUFA, particularly diets rich in fish oil, may result in immunosuppression *in vivo*. Indeed, three studies have reported that feeding laboratory rodents fish oil results in diminished *in vivo* graft vs. host and/or host vs. graft responses (41–43); feeding diets rich in n-6 PUFA did not suppress these responses (43).

Thus, it is now quite clear that consumption of diets rich in n-3 PUFA causes marked immunosuppression, whereas those rich in n-6 PUFA are less suppressive. However, there is little information about how much n-3 PUFA, particularly in relation to n-6 PUFA, needs to be present to bring about these effects. Therefore, in this study we used mixtures of sunflower and linseed oils to create a series of diets with differing ratios of n-6 to n-3 PUFA. We have investigated the effect of feeding these diets to rats upon serum lipid levels and fatty acid composition, upon spleen lymphocyte fatty acid composition and functions, and upon the graft vs. host response.

MATERIALS AND METHODS

Animals and diets. Weanling male Lewis rats (aged three weeks, weighing between 65 and 85 g) were obtained from Harlan-Olac (Bicester, Oxfordshire, England). The animals were housed in the Department of Biochemistry at the University of Oxford (Oxford, United Kingdom) for a period of six weeks prior to sacrifice, during which time they were allowed access, *ad libitum*, to water and to one of five experimental diets. The diets contained 20% by weight of sunflower oil or linseed oil or mixtures of the two designed to yield different ratios of n-6 to n-3 PUFA; the levels of other components of the diets were identical. It is important, to note that the proportions of fatty acids other than linoleic and α-linolenic were not different among the different diets. The ratios of n-6 to n-3 PUFA used in this study were 112.5:1 (all sunflower oil), 14.8:1 (93% sunflower oil), 6.5:1 (85% sunflower oil), 0.8:1 (30% sunflower oil), and 0.33:1 (all linseed oil). The ratio of 6.5:1 was chosen to approximate the n-6 to n-3 PUFA ratio of the average United Kingdom diet, which is 6.9:1 (44). The ratio of 14.8:1 was chosen as it approximates the upper extreme of the n-6 to n-3 PUFA ratio found in Western societies. For example, the average n-6 to n-3 PUFA ratio of human breast milk in the United Kingdom is 11:1, and some infant formula have ratios as high as 15:1 (44). The ratio of 0.8:1 was chosen so a diet with approximately equal proportions of n-6 and n-3 PUFA could be investigated. The diets used in this study contain at least ten times more fatty acid double bonds per g than standard laboratory chow, and it was considered important to include a significantly higher level of vitamin E than would normally be present in order to prevent fatty acid oxidation. Therefore, these diets were supplemented with 300 IU of vitamin E per kg, ten times the recommended level. The composition of the diets is shown in Table 1 and the fatty acid composition is shown in Table 2. Diets were stored at 4°C and were provided fresh to the animals every two days. Rats were killed in the fed state by an overdose of CO_2.

Chemicals. Chemicals and cell culture medium and supplements were obtained from the sources described elsewhere (8,13,40,43,45).

Cholesterol, TAG, and nonesterified fatty acid (NEFA) measurements. Cholesterol and TAG concentrations were determined enzymatically (46,47). NEFA concentrations were measured using a commercially available enzymatic assay kit (Boehringer, Mannheim, Germany).

Lipid extraction and fatty acid composition analysis. Lipid was extracted from serum or from lymphocytes with chloroform/methanol (2:1, vol/vol). Fatty acids were prepared by overnight saponification at 70°C in methanolic 0.5 M KOH.

TABLE 1
Composition of the Diets Used

Component (g/kg)	Diet A	B	C	D	E
High nitrogen casein	200	200	200	200	200
Corn starch	200	200	200	200	200
Sucrose	295.8	295.8	295.8	295.8	295.8
Nonnutritive bulk	50	50	50	50	50
Mineral mix[a]	40	40	40	40	40
DL-α-Tocopherol[b]	1.2	1.2	1.2	1.2	1.2
Methionine	3	3	3	3	3
Vitamin mix[c]	10	10	10	10	10
Sunflower oil	200	188	176	74	0
Linseed oil	0	12	24	126	200

[a]AIN-76 containing per kg: 500 g calcium phosphate dibasic, 74 g sodium chloride, 220 g potassium citrate monohydrate, 52 g potassium sulfate, 24 g magnesium oxide, 3.5 g manganese carbonate, 6 g ferric citrate, 1.6 g zinc carbonate, 0.3 g cupric carbonate, 0.01 g potassium iodate, 0.01 g sodium selenite, 0.55 g chromium potassium sulfate, 118 g sucrose.
[b]250 IU/g.
[c]Containing per kg: 1.8 g vitamin A (500,000 IU/g), 0.125 g vitamin D2 (850,000 IU/g), 22 g DL-α-tocopherol, 45 g ascorbic acid, 5 g inositol, 75 g choline chloride, 2.25 g menadione, 5 g *p*-aminobenzoic acid, 4.25 g niacin, 1 g riboflavin, 1 g pyridoxine hydrochloride, 1 g thiamine hydrochloride, 3 g calcium pantothenate, 0.02 g biotin, 0.09 g folic acid, 0.00135 g vitamin B12, 833.46 g carrier dextrose.

TABLE 2
Fatty Acid Composition of the Diets Used

	Fatty acid (mol%)							
Diet	14:0	16:0	16:1n-7	18:0	18:1n-9	18:2n-6	18:3n-3	n-6/n-3
A	0.1	8.0	0.4	4.1	19.1	67.5	0.6	112.5
B	0.1	8.5	0.5	4.5	21.7	58.4	4.0	14.75
C	0.1	7.4	0.4	4.6	21.8	57.7	8.9	6.47
D	0.1	8.1	0.5	4.6	21.8	29.2	35.6	0.82
E	0.1	7.6	0.5	4.6	22.0	16.3	48.8	0.33

Samples were neutralized using concentrated sulfuric acid, and fatty acids were extracted into ethyl acetate. After evaporation to dryness, fatty acids were prepared by reaction with an excess of diazomethane in ether (48). Fatty acid methyl esters (dissolved in methyl acetate) were separated by gas chromatography in a Hewlett-Packard (Avondale, PA) 6890 gas chromatograph fitted with a 30 m × 0.32 mm DB23 capillary column (J&W Scientific, Folsom, CA), film thickness 0.25 mm. Nitrogen at 2.0 mL/min was used as the carrier gas and the split/splitless injector was used with a split/splitless ratio of 10:1. Injector and detector temperatures were 170 and 250°C, respectively. The column oven temperature was maintained at 170°C for 12 min after sample injection, and was programmed to then increase from 170 to 200°C at 5°C/min before being maintained at 200°C for 15 min. The separation was recorded with HP GC Chem Station software (Hewlett-Packard). Fatty acid methyl esters were identified by comparison with previously run standards.

Lymphocyte proliferation. Spleen lymphocytes were prepared as described elsewhere (13). They were cultured at 37°C in an air/CO_2 (19:1) atmosphere in 96-well micro-titer-culture plates at a density of 5×10^5 cells/well and a total culture volume of 200 µL in HEPES-buffered (RPMI) supplemented with 2 mM glutamine, 2.5% (vol/vol) autologous serum, antibiotics (streptomycin and penicillin) and, for stimulated lymphocytes, 5 µg/mL concanavalin A (Con A). After 48 h of culture, [6-^{3}H]thymidine was added to each well (0.2 µCi/well), and the cells were incubated for a further 18 h. The cells were then harvested onto glass fiber filters and washed and dried using a Skatron Cell Harvester (Skatron Instruments, Lier, Norway). Radioactive thymidine incorporation was determined by liquid scintillation counting. The results are expressed as a stimulation index:

$$\text{stimulation index} = \frac{\text{incorporation of [}^3\text{H]thymidine in the presence of Con A}}{\text{incorporation of [}^3\text{H]thymidine in the absence of Con A}} \quad [1]$$

Spleen lymphocyte NK cell activity. Spleen lymphocytes (100 µL) suspended in RPMI supplemented with 2 mM glutamine and YAC-1 cells (2×10^4 cells/100 µL) suspended in RPMI supplemented with 2 mM glutamine and 1% (wt/vol) bovine serum albumin were added, in triplicate, to each well of a 96-well, flat-bottomed micro-titer plate to yield lymphocyte/target cell ratios of 100:1, 50:1, and 25:1. Plates were centrifuged at 300 rpm for 3 min to bring lymphocytes and target cells into contact, and were then incubated at 37°C in an air/CO_2 (19:1) atmosphere for four hours. The plates were centrifuged at 500 rpm for 5 min after the incubation. Target cell killing was determined by release of lactate dehydrogenase (LDH) into the medium, as measured by a "cytotoxicity assay kit" purchased from Proteins International Inc. (Rochester Hills, MI). Maximal LDH release was determined by incubating YAC-1 cells with 2.5% (vol/vol) Triton X-100 in RPMI, instead of the lymphocytes. Spontaneous LDH release from YAC-1 cells was determined by incubating them with glutamine- and antibiotic-supplemented RPMI in place of the lymphocytes. Spontaneous LDH release from lymphocytes was determined by incubating them at the appropriate cell number with glutamine-, albumin; and antibiotic-supplemented RPMI in place of the YAC-1 cells. Percentage cytolysis was calculated as:

$$\% \text{ cytolysis} = \frac{\text{LDH release - lymphocyte spontaneous LDH release - YAC-1 spontaneous LDH release}}{\text{maximum LDH release - YAC-1 spontaneous LDH release}} \times 100 \quad [2]$$

Analysis of lymphocyte subpopulations. Flow cytometry was used to measure the presence of various markers on the surface of freshly-prepared spleen lymphocytes. Approximately 10^6 cells [resuspended in phosphate-buffered saline (PBS) supplemented with 0.1% (wt/vol) bovine serum albumin and 10 mM sodium azide (modified PBS)] were incubated for 20 min at 4°C with monoclonal antibodies to CD4 (W3/25), CD8 (MRC OX-8), κ-light chain of immunoglobulins (MRC OX12; a B-cell marker), CD11b/c (MRC OX42; a macrophage marker), or CD16 (3.2.3; an NK cell marker). Incubation with a monoclonal antibody to the human C3b inactivator protein (MRC OX-21) was used as a negative control. Following staining with monoclonal antibodies, the cells were washed twice with modified PBS and incubated with fluorescein isothiocyanate-labelled rabbit anti-mouse IgG for 20 min at 4°C. They were washed twice with modified PBS and then suspended in 2% (vol/vol) formaldehyde in PBS and examined for fluorescence using a Becton Dickinson FACScan fluorescence-activated cell sorter (Becton Dickinson, Lincoln Park, NJ). Fluorescence data were collected on 10^4 viable cells.

Popliteal lymph node (PLN) assay. To elicit the graft vs. host response, 6×10^6 lymph node lymphocytes prepared from adult male Lewis rats (maintained on standard laboratory chow) were injected subcutaneously into the footpad of one leg of weanling male DA/Lewis rats which had been fed the diets previously described, for four weeks. The total in-

jection volume was 0.1 mL, and the cells were resuspended in PBS; the footpad of the control leg was injected with 0.1 mL PBS. The animals were sacrificed seven days after injection; during this time they were fed the same diet that they had been previously fed. The PLN were dissected out and weighed. Preliminary experiments indicated that injection of 6×10^6 cells and sacrifice 7 d post-injection resulted in maximal PLN size (43). Injection of PBS or of 6×10^6 DA/Lewis lymph node lymphocytes did not cause any increase in PLN weight (43).

Data presentation. Data are mean ± SEM of separate observations made on the indicated number of animals fed each diet. Statistical significance was determined using one-way analysis of variance followed by Student's *t*-test; a value for *P* of less than 0.05 was taken to indicate a statistically significant difference.

RESULTS

Animal growth. The diets used in this study were isocaloric, and there was no difference in food intake between animals fed the different diets (data not shown). Despite this, the final body weights, and therefore the total growth, of rats fed diet E were less than those of rats fed diets B and C (Table 3). Seven distinct adipose tissue depots were dissected out and weighed; the sum of these weights was termed the total dissectable fat. Total dissectable fat was less in animals fed diet E than in those fed diets A, B, or C (Table 3). Total dissectable fat accounted for a smaller percentage of body weight in the animals fed diet E than those fed diets A or B (Table 3). Thus, feeding the 20% linseed oil diet reduced adipose deposition compared with a diet containing 20% sunflower oil or diets containing more linoleic acid than α-linolenic acid. There was little difference in liver weight between animals fed the different diets (Table 3).

Serum cholesterol, TAG, and NEFA concentrations. The concentrations of TAG, cholesterol, and NEFA all progressively decreased as the n-6/n-3 ratio of the diet decreased (Table 4). The TAG concentration in the serum of animals fed the diet with the lowest n-6/n-3 ratio (diet E) was 40% of that of animals fed the diet with the highest ratio (diet A). The cholesterol concentration in the serum of rats fed the diet with the lowest n-6/n-3 ratio was significantly less than that in the serum of animals fed the diets with the three highest ratios (Table 4). The NEFA concentration in the serum of rats fed diets C, D, or E was significantly lower than that in the serum of animals fed diet A (Table 4).

Serum and spleen lymphocyte fatty acid composition. The fatty acid composition of the serum was influenced by that of the diet (Table 5). As the n-6/n-3 PUFA ratio of the diet decreased, the proportion of n-6 PUFA (linoleic, arachidonic) in serum lipids progressively decreased. This was parallelled by a progressive increase in the proportion of α-linolenic acid (Table 5). Eicosapentaenoic acid was found in the serum of animals fed the diets which were richest in n-3 PUFA (diets D and E), and docosahexaenoic acid was present in the serum of all animals fed n-3 PUFA-containing diets (Table 5). The proportions of the other principal serum fatty acids (palmitic, stearic, oleic) were largely unaffected by the diet fed (Table 5).

The fatty acid composition of the lipids of spleen lymphocytes was also affected by that of the diets fed (Table 5). As the n-6/n-3 PUFA ratio of the diet decreased, the proportion of n-6 PUFA (linoleic, arachidonic) in lymphocyte lipids progressively decreased. This was parallelled by a progressive increase in the proportion of α-linolenic acid (Table 5). Despite the incorporation of significant levels of α-linolenic acid, neither eicosapentaenoic nor docosahexaenoic acid was detected in spleen lymphocyte lipids (Table 5).

Proliferation of spleen lymphocytes. Feeding each of the diets containing linseed oil (i.e., diets B, C, D, or E) significantly decreased Con A-stimulated proliferation of spleen lymphocytes compared with feeding diet A (Fig. 1). There was no difference in the proliferation of spleen lymphocytes from rats fed the diets containing linseed oil (Fig. 1).

Spleen NK cell activity. Feeding each of the diets containing linseed oil (i.e., diets B, C, D, or E) decreased spleen lymphocyte NK activity compared with feeding diet A (Table 6).

TABLE 3
Effect of Feeding Diets of Different Fatty Acid Compositions upon Animal and Liver Weights[a]

Diet	Initial body weight (g)	Final body weight (g)	Total dissectable fat (g)	Liver weight (g)
A	45.0 ± 2.4	296.0 ± 10.7	24.9 ± 2.0[e]	12.7 ± 0.3[b]
			(8.4 ± 0.5[e])	(4.3 ± 0.1)
B	44.2 ± 2.2	317.0 ± 8.4[e]	27.1 ± 1.7[e]	13.9 ± 0.2[a]
			(8.3 ± 0.4[e])	(4.4 ± 0.1)
C	45.0 ± 3.1	310.0 ± 9.7[e]	26.0 ± 2.2[e]	13.6 ± 0.5
			(8.4 ± 0.7)	(4.4 ± 0.2)
D	50.0 ± 4.3	306.0 ± 6.9	22.2 ± 1.3	13.8 ± 0.6
			(7.2 ± 0.3)	(4.5 ± 0.1)
E	42.5 ± 1.6	281.0 ± 7.1[b,c]	18.9 ± 0.8[a,b,c]	13.2 ± 0.6
			(6.9 ± 0.2[a,b])	(4.7 ± 0.1)

[a]Data are mean ± SEM of separate observations made on six animals fed each diet. Numbers in parentheses are total dissectable fat or liver weight as a percentage of final body weight. [a]vs. Diet A; [b]vs. diet B; [c]vs. diet C; [d]vs. diet D, and [e]vs. diet E.

TABLE 4
Effect of Feeding Diets of Different Fatty Acid Compositions Upon Serum Lipid Levels[a]

Diet	Cholesterol (mg/mL)	TAG (mg/mL)	NEFA (mM)
A	1.20 ± 0.06[e]	0.88 ± 0.12[e]	0.67 ± 0.02[c,d,e]
B	1.21 ± 0.05[e]	0.62 ± 0.08[e]	0.56 ± 0.04
C	1.14 ± 0.04[e]	0.73 ± 0.12[e]	0.51 ± 0.03[a]
D	1.08 ± 0.04	0.50 ± 0.10	0.54 ± 0.04[a]
E	0.93 ± 0.04[a,b,c]	0.36 ± 0.05[a,b,c]	0.50 ± 0.03[a]

[a]Data are mean ± SEM of separate observations made on six animals fed each diet. Superscript letters a, b, c, d, e are explained in Table 3. TAG, triacylglycerol; NEFA, nonesterified fatty acid.

There was no difference in the NK cell activity of spleen lymphocytes from rats fed the diets containing linseed oil, except at a lymphocyte/target cell ratio of 25:1 (Table 6).

Spleen lymphocyte subsets. There was no effect of diet upon the proportions of cells staining positive for CD4, CD8, κ-light chain, or CD11b/c (Table 7). Thus, changing the n-6/n-3 ratio of the diet does not differentially affect the proportions of $CD4^+$, $CD8^+$, or B-cells or macrophages in the spleen. However the proportion of $CD16^+$ cells (i.e., NK cells) was affected by diet; the proportion of these cells was higher in the spleens of animals fed diets D and E than in those of animals fed some of the other diets (Table 7).

PLN weights. The weight of the PLN following the graft vs. host response progressively decreased as the n-6/n-3 ratio of the diet decreased (Fig. 2). Weights of PLN from animals fed diet E were 35% lower than those from animals fed diet A. Weights of PLN from rats fed diets D and E were significantly lower than those from animals fed diet A. In addition, PLN weights from animals fed diet E were lower than those from the diet B-fed animals (Fig. 2).

DISCUSSION

Compared with feeding rats diets rich in n-6 PUFA, diets rich in fish oil result in lower serum cholesterol (4,8) and TAG (7) concentrations. Linseed oil also has been reported to result in a lower serum cholesterol concentration when fed to rats (49). In accordance with these observations, we found that feeding rats diets containing linseed oil resulted in lower serum cholesterol and TAG concentrations than observed after feeding a sunflower oil-rich diet (Table 4). The serum cholesterol concentration of rats fed 20% linseed oil (i.e., diet E) was higher than that which we had previously found following 20% fish oil feeding (0.71 mg/mL; Ref. 8), whereas the serum TAG concentration was lower (0.77 mg/mL; Ref. 8). Thus, in rats, linseed oil may be more effective than fish oil at lowering TAG concentrations, but less effective at lowering cholesterol concentrations. Despite these differences, it is clear that n-3 PUFA have a blood lipid-lowering effect in rats whether they are derived from fish or linseed oils. This effect of n-3 PUFA may be due to suppressed lipoprotein synthesis and/or secretion or to enhanced clearance of circulating lipoproteins or both. In agreement with the observations of Rustan *et al.* (50), the linseed oil-containing diets also resulted in lowered serum

TABLE 5
Effect of Feeding Diets with Different n-6/n-3 Fatty Acid Ratios upon Serum and Spleen Lymphocyte Fatty Acid Composition[a]

Tissue	Diet	Fatty acid (mol %) 14:0	16:0	16:1n-7	18:0	18:1n-9
Serum	A	0.9 ± 0.1	22.1 ± 0.5[d,e]	2.9 ± 0.5	20.9 ± 1.5	9.1 ± 0.3
	B	0.8 ± 0.1	21.4 ± 1.6[d,e]	2.7 ± 0.7	21.9 ± 1.4	7.8 ± 0.6
	C	0.9 ± 0.2	22.9 ± 0.4[d,e]	2.9 ± 0.6	20.1 ± 1.2	8.5 ± 0.5
	D	0.9 ± 0.1	26.1 ± 1.0[a,b,c]	2.8 ± 0.9	22.4 ± 1.1	9.9 ± 0.8
	E	0.8 ± 0.1	26.3 ± 1.1[a,b,c]	4.1 ± 0.3	22.6 ± 1.4	9.1 ± 0.3
Lymphocyte	A	2.3 ± 0.3[b,c,e]	26.4 ± 1.0	3.6 ± 0.8[e]	20.4 ± 1.9	9.8 ± 0.9[d]
	B	3.2 ± 0.2[a,c,e]	23.7 ± 1.5[d]	3.1 ± 0.6[e]	17.3 ± 0.9	9.8 ± 0.8[d]
	C	4.9 ± 0.3[a,b]	27.0 ± 1.8	4.0 ± 0.1[e]	19.2 ± 0.6[e]	11.7 ± 0.2
	D	3.3 ± 0.7	29.2 ± 1.1[b]	3.1 ± 0.7[e]	19.3 ± 0.7[e]	12.7 ± 0.7[a,b,e]
	E	4.4 ± 0.4[a,b]	31.4 ± 3.6	6.6 ± 0.3[a,b,c,d]	16.1 ± 1.1[c,d]	8.6 ± 1.4[d]
		18:2n-6	18:3n-3	20:4n-6	20:5n-3	22:6n-3
Serum	A	31.6 ± 1.2[d,e]	n.d.	11.6 ± 0.5[c,d,e]	n.d.	n.d.
	B	30.8 ± 0.9[d,e]	0.6 ± 0.1[c,d,e]	10.5 ± 1.0[d,e]	n.d.	1.3 ± 0.1[d,e]
	C	30.1 ± 1.0[d,e]	1.2 ± 0.1[b,d,e]	10.0 ± 0.5[a,d,e]	n.d.	1.4 ± 0.1[d,e]
	D	21.9 ± 1.5[a,b,c]	8.6 ± 0.2[b,c,e]	7.0 ± 0.5[a,b,c,e]	0.7 ± 0.1[e]	0.8 ± 0.1[b,c]
	E	20.2 ± 0.4[a,b,c]	11.6 ± 1.0[b,c,d]	4.6 ± 0.5[a,b,c,d]	1.4 ± 0.2[d]	0.8 ± 0.1[b,c]
Lymphocyte	A	30.0 ± 0.6[b,c,d,e]	n.d.	12.1 ± 0.6[c,d,e]	n.d.	n.d.
	B	27.9 ± 0.4[a,c,d,e]	0.4 ± 0.1[c,d,e]	14.3 ± 1.0[c,d,e]	n.d.	n.d.
	C	22.9 ± 1.5[a,b,e]	2.6 ± 0.3[b,d,e]	7.3 ± 0.3[a,b]	n.d.	n.d.
	D	20.9 ± 1.7[a,b]	5.7 ± 0.6[b,c,e]	8.3 ± 0.7[a,b,e]	n.d.	n.d.
	E	18.1 ± 0.9[a,b,c]	8.0 ± 0.3[b,c,d]	5.0 ± 1.3[a,b,d]	n.d.	n.d.

[a]Data are mean ± SEM for separate observations made on at least six animals fed each diet; n.d. indicates that a fatty acid was not detected; a, b, c, d, e, are explained in Table 3.

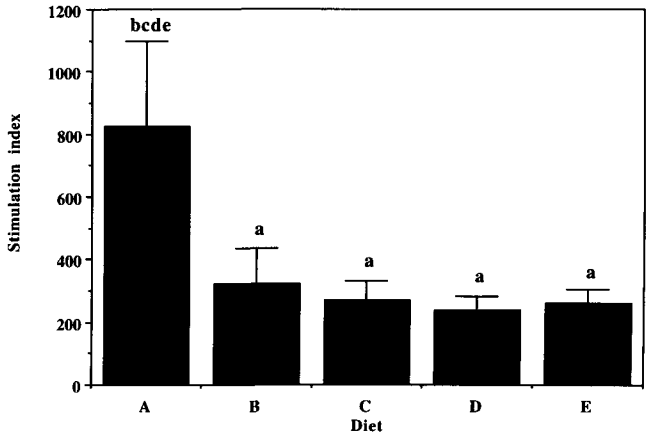


FIG. 1. Effect of feeding diets of different fatty acid compositions upon the proliferation of spleen lymphocytes. Lymphocytes were cultured in autologous serum in the absence or presence of concanavalin A; lymphocyte proliferation is expressed as stimulation index (see the Materials and Methods section). Data are mean ± SEM of separate observations made on three to five animals fed each diet. a vs. Diet A, b vs. diet B, c vs. diet C, d vs. diet D, and e vs. diet E.

NEFA concentrations (Table 4). This may indicate that oxidation of fatty acids is enhanced in these animals. α-Linolenic acid is a better substrate for β-oxidation than linoleic acid (51), and so its inclusion in the diet at the expense of linoleic acid may promote fatty acid oxidation. Furthermore, like the n-3 PUFA found in fish oil (50,52), dietary α-linolenic acid promotes hepatic peroxisomal proliferation (50) and this, in turn, will lead to enhanced oxidation of fatty acids. As a result of the enhanced fatty acid oxidation, less fatty acid will be available for hepatic TAG synthesis, and so circulating lipid levels will be reduced. The lowered circulating TAG level means that less TAG is available for deposition in adipose tissue. Indeed, the total dissectable fat was significantly reduced in animals fed diet E (Table 3). This decrease in adipose deposition, which we have reported previously for animals fed fish oil (53), partially accounts for the lower weight of animals fed diet E.

It has previously been shown that feeding rats a diet containing 20% fish oil results in suppressed *ex vivo* lymphocyte proliferation (13,29,30) and NK cell activity (29,40) and diminishes the graft vs. host response *in vivo* (43). Similar effects of fish oil feeding have been reported in other species, including mice (28,36,38,39,42), rabbits (25), chickens (26), and humans (54–56). Feeding linseed oil to laboratory animals also has been reported to suppress lymphocyte proliferation *ex vivo* (24–26); this effect was confirmed in the current study (Diet E of Fig. 1). This suppressive effect is consistent with the potent inhibitory effects of α-linolenic acid and of TAG rich in α-linolenic acid upon lymphocyte proliferation *in vitro* (14-21). An effect of dietary linseed oil upon NK cell activity, as observed in the current study (Table 6), has not been previously reported. Furthermore, it was found that a 20% linseed oil diet diminished the graft vs. host response (Diet E of Fig. 2), an *in vivo* measurement of cell-mediated immunity. We now report, also for the first time, that the inclusion of a small amount of linseed oil in a diet rich in n-6 PUFA is sufficient to bring about these suppressive effects.

The maximal effect of dietary α-linolenic acid upon *ex vivo* proliferation of spleen lymphocytes (a reduction by 65%) was observed with diet B; increasing the amount of α-

TABLE 6
Effect of Feeding Diets of Different Fatty Acid Compositions upon Spleen Lymphocyte NK Cell Activity[a]

Diet	% Cytolysis (L/T)		
	100:1	50:1	25:1
A	28.2 ± 1.8^{b,d,e}	19.9 ± 2.1^{d,e}	10.7 ± 1.6^{d,e}
B	22.0 ± 1.8^{a}	13.6 ± 2.0	11.1 ± 1.6^{d,e}
C	22.7 ± 1.8	15.4 ± 0.8	9.7 ± 0.5^{d,e}
D	18.6 ± 0.6^{a}	12.9 ± 1.1^{a}	6.8 ± 0.7^{a,b,c}
D	19.7 ± 1.7^{a}	13.1 ± 1.6^{a}	6.5 ± 0.9^{a,b,c}

[a]Natural killer (NK) cell activity was determined at different lymphocyte to target cell ratios (L/T) as described in the Materials and Methods section. Data are mean ± SEM of separate observations made on five animals fed each diet. Superscript letters a, b, c, d, e are explained in Table 3.

TABLE 7
Effect of Feeding Diets of Different Fatty Acid Compositions upon Spleen Lymphocyte Subpopulations[a]

Diet	Percentage of positive cells				
	CD4	CD8	κ-Light chain	CD11b/c	CD16
A	53.9 ± 2.0	34.9 ± 1.4	37.1 ± 1.6	22.0 ± 0.9	6.7 ± 0.6^{d}
B	52.2 ± 2.5	35.0 ± 3.8	39.2 ± 2.0	28.2 ± 4.4	6.1 ± 0.7^{d}
C	53.4 ± 1.9	33.3 ± 0.6	40.9 ± 1.3	23.0 ± 3.4	5.8 ± 0.4^{d,e}
D	53.0 ± 1.5	36.5 ± 1.7	41.5 ± 2.9	26.2 ± 3.5	9.3 ± 0.9^{a,b,c}
E	51.3 ± 2.4	33.5 ± 1.3	42.1 ± 1.7	23.9 ± 3.8	8.0 ± 0.9^{c}

[a]The proportion of cells expressing each marker was determined as described in the Materials and Methods section. Data are mean ± SEM of separate observations made on five animals fed each diet. Superscript letters a, b, c, d, e, are explained in Table 3.

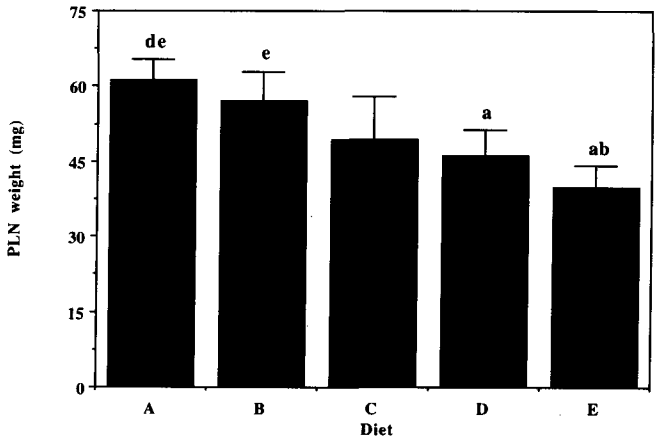


FIG. 2. Effect of feeding diets of different fatty acid compositions upon the graft vs. host response. Lymphocytes from Lewis rats were injected into the footpad of DA/Lewis rats (see the Materials and Methods section). Seven days later the popliteal lymph nodes (PLN) were dissected out and weighed. Data are mean ± SEM of separate observations made on 10 to 12 animals fed each diet; a, b, d, e, are explained in Figure 1.

linolenic acid had no further effect (Fig. 1). It is likely that a progressive effect of dietary α-linolenic acid upon lymphocyte proliferation would have been observed had a diet with an n-6 to n-3 PUFA ratio between those of diets A and B been used. The n-6 to n-3 PUFA ratio of such a diet would have been greater than 15 and would have been much higher than that consumed in the human diet.

The n-3 PUFA-induced inhibition of lymphocyte function did not involve a change in the proportions of $CD4^+$ cells, $CD8^+$ cells, B-cells, or macrophages in the spleens of these animals (Table 7). However, linseed oil feeding did increase the proportion of $CD16^+$ cells (i.e., NK cells) in the spleen (Table 7). Therefore, the diminished NK cell activity of spleen lymphocytes was not due to there being less NK cells present. Indeed, as there were more NK cells present, but lower overall NK cell activity, activity per NK cell must have been significantly decreased in rats fed diets D and E. This effect may be explained by the lowered arachidonic acid content of lymphocytes from animals fed diets C, D, and E. The arachidonic acid-derived eicosanoid leukotriene (LT) B_4 is a potent activator of NK cell activity (57), and it is known that feeding linseed oil diminishes the production of 4-series LT (58). Thus, diminished production of LTB_4 in cultures of lymphocytes isolated from animals fed diets C, D, and E may account for the decreased NK cell activity observed (Table 6).

The n-6/n-3 ratio of the 20% fish oil diet used in our previous experiments (8,13,28–30,40,43,45,52,53) was 0.3:1, very similar to the ratio of the 20% linseed oil diet used in the present study (diet E). The effects observed after feeding these two diets are very similar. As indicated previously, both diets result in significant lowering of blood lipid levels. Furthermore, both diets result in significant suppression of *ex vivo* spleen lymphocyte proliferation and NK cell activity and of the graft vs. host response, an *in vivo* measure of cell-mediated immunity; the effects of the linseed oil diet (Figs. 1 and 2, Table 6) are as strong as those of the fish oil diet (13,40,43). However, in contrast to fish oil feeding (45), the linseed oil diet did not result in significant incorporation of eicosapentaenoic and docosahexaenoic acids into spleen lipids (Table 5). This was despite the appearance of these long-chain n-3 PUFA in the serum of linseed oil-fed animals. These observations indicate that the immunomodulatory actions of n-3 PUFA are not restricted to those present in high proportions in fish oil (i.e., eicosapentaenoic and docosahexaenoic acids), but that plant oils rich in n-3 PUFA (e.g., linseed oil) also exert potent effects. These effects were elicited when only relatively small amounts of linseed oil were added to sunflower oil. For example, the inhibition of *ex vivo* spleen lymphocyte proliferation (Fig. 1) and NK cell activity (Table 6) were observed following the feeding of diet B; this inhibition did not increase as the n-3 PUFA content of the diet increased. The effect upon cell-mediated immunity *in vivo* (i.e., the graft vs. host response) did increase as the α-linolenic acid content of the diet increased (Fig. 2).

In conclusion, this study indicates that dietary n-3 PUFA other than those found in fish oils elicit potent effects upon blood lipid levels and immune cell function in the rat, and that only a relatively small amount of such fatty acids need to be added to the diet to bring about these effects. It will be important to find out whether similar linseed oil-induced immunoregulatory effects occur in humans. Fish oil consumption has been suggested as a means for lowering blood lipid levels in man and to offer protection against the development of CHD and inflammatory disorders. Because α-linolenic acid is available from plant sources and as it is less susceptible to oxidative damage than the longer chain n-3 PUFA, it may be a useful alternative to fish oil.

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Differential Alterations of Ethanolamine and Choline Phosphoglyceride Metabolism by Clofibrate and Retinoic Acid in Human Fibroblasts Are Not Mediated by Phorbol Ester-Sensitive Protein Kinase C

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ABSTRACT: Peroxisomal proliferators and retinoids have been reported to interact to regulate lipid metabolism, particularly β-oxidation of fatty acids. Based on postulated interactions of these agents at the levels of receptors and response elements, we examined whether interactions exist between the peroxisomal proliferator, clofibrate (CLF), and retinoic acid (RA) in modulation of phospholipid turnover in cultured human skin fibroblasts. Treatment of cultured cells with either 25 μM CLF or 1 μM RA alone decreased [^{14}C]ethanolamine incorporation into ethanolamine phosphoglycerides (EPG) by 20–30%, and simultaneous exposure to both agents resulted in additive inhibition. By contrast, [^{3}H]choline incorporation into phospholipid was stimulated 5–30% by incubation with either agent; when CLF and RA were administered together, the stimulatory effects were additive. Different types of pulse–chase studies examining effects on uptake, biosynthesis, and degradation of labelled phospholipids indicated stimulation of EPG degradation and inhibition of phosphatidylcholine degradation by CLF; no effect on catabolism of either phospholipid was observed with RA. Combinations of modifiers of protein kinase activity [4β-12-*O*-tetradecanoylphorbol-13-acetate (β-TPA), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride, *N*-(2′-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride, *bis*-indolylmaleimide, staurosporine] indicated that β-TPA-responsive protein kinases were not involved. Accordingly, CLF and RA regulate biosynthesis and degradation of ethanolamine and choline phosphoglycerides in cultured skin fibroblasts by different mechanisms that do not involve classical protein kinase C (PKC) isoforms, even though turnover of phospholipids generating lipid activators of PKC occurs.
Lipids 31, 747–755 (1996).

Clofibrate (CLF) is a hypolipidemic drug commonly used in the treatment of patients with hypertriglyceridemia (1). This agent also is known for its properties as a peroxisomal proliferator in rodent species, where dietary intake for several weeks causes marked increases in peroxisomal size, number, and concomitant induction of peroxisome-associated enzymes, particularly catalase and those catalyzing β-oxidation of fatty acids (2,3). Other effects on lipid metabolism not specifically involving peroxisomes include alterations of mitochondrial enzyme activities and intracellular transport by fatty acid binding proteins (4,5). Some of the diverse effects of CLF appear to be mediated by peroxisomal proliferator-activated receptors (PPAR). These members of the steroid/thyroid hormone superfamily of ligand-activated transcription factors recently have been cloned and characterized (6,7).

Retinoic acid (RA), a naturally-occurring metabolite of vitamin A (retinol), is an important regulator of cellular proliferation, differentiation, and metabolism with a wide range of effects in many cells and tissues (8,9). Most, if not all, biological actions of RA are thought to be mediated by receptors (e.g., RAR and RXR) which, like the PPAR, have been shown to belong to the steroid hormone receptor superfamily (10–12). Retinoids can interact with peroxisomal proliferators, presumably *via* their receptors, to modulate lipid metabolism, specifically, β-oxidation of fatty acids (13–15).

Previous studies from our laboratory showed that CLF inhibits turnover of ethanolamine phosphoglycerides (EPG) in cultured human skin fibroblasts with selectivity relative to its effects on metabolism of choline phosphoglycerides (16). As recent reports have described interactions between peroxisomal proliferators and retinoids in the regulation of lipid metabolism and receptors for these two agents interact in the regulation of gene expression (13–15), the present study was initiated to determine whether clofibrate and retinoic acid might interact to coordinately or synergistically alter phosphoglyceride turnover in cultured human skin fibroblasts. We provide evidence supporting differential regulation of EPG and choline phosphoglyceride synthesis and catabolism by CLF and RA. As stimulators of protein kinase C (PKC) also alter phospholipid turnover (17–19) and PKC may be involved in

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Abbreviations: CLF, clofibrate; DMSO, dimethylsulfoxide; EPG, ethanolamine phosphoglycerides; HA-1004, *N*-(2′-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; MEM, Eagle's minimal essential medium; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PMSF, phenylmethylsulfonylfluoride; PPAR, peroxisomal proliferator-activated receptor(s); PtdCho, phosphatidylcholine; RA, retinoic acid; β-TPA, β-12-*O*-tetradecanoylphorbol-13-acetate.

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some actions of CLF and RA (20–22), we investigated whether PKC might participate in the actions of these agents. Our results suggest that phorbol ester-sensitive isoforms of PKC do not initiate or mediate the differential effects of CLF and RA on phospholipid metabolism, even though turnover of these classes of phospholipid can potentially supply lipid activators of PKC, particularly diacylglycerol species for sustained activation (23).

MATERIALS AND METHODS

Materials. [methyl-^{3}H]Choline (86.6 Ci/mmol) and [γ-^{32}P]ATP were purchased from DuPont Canada, Inc. (NEN Research Products, Montreal, Quebec, Canada). [1,2-^{14}C]Ethanolamine hydrochloride (11.7 mCi/mmol), unlabelled choline, RA, 2-(*p*-chlorophenoxy)-2-methyl-propionic acid ethyl ester (CLF), dimethylsulfoxide (DMSO), phenylmethylsulfonylfluoride (PMSF), leupeptin, digitonin, forskolin, dideoxyforskolin, and bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO). *Bis*-indolylmaleimide hydrochloride was purchased from Calbiochem-Novabiochem Corporation (LaJolla, CA), 1-(5-isoquinolinsulfonyl)-2-methylpiperazine dihydrochloride (H-7), *N*-(2′-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride (HA-1004), staurosporine, and 4β-12-*O*-tetradecanoylphorbol-13-acetate (β-TPA) were from LC Laboratories (Woburn, MA). Phosphatidylserine was from Serdary Research Laboratories Inc. (London, Ontario, Canada). All other chemicals and reagents were of high quality and obtained from commercial sources. PKC assay kits were from Amersham Canada Ltd. (Oakville, Ontario, Canada).

Cell culture. Human skin fibroblasts (F8) were subcultured, as described previously (16,24), in 60-mm plastic dishes in 5 mL Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum. Cultures were maintained at 37°C, under 95% air/5% CO_2, for 7 to 8 d. Protein content ranged from 0.4–0.6 mg per dish at initiation of experiments.

Cell incubation. All incubations were carried out at 37°C. For pulse experiments, confluent cultures were placed in 2 mL fresh MEM without fetal bovine serum and incubated for 24 h. Appropriate reagents and labelled substrate [either [^{14}C]ethanolamine (65 μM; 1.5 μCi) or [^{3}H]choline (7 μM; 3 μCi)] were added to each dish and incubated for the indicated times. All reagents were dissolved in DMSO (final concentration not exceeding 1%), except for H-7 and HA-1004, which were dissolved in water. Cells were rinsed twice with 2 mL phosphate-buffered saline at pH 7.4, harvested by scraping in methanol/water (5:4, vol/vol), and disrupted by sonication (2 × 10 s bursts). Aliquots were taken for protein determination (25) using bovine serum albumin as standard. Lipids were extracted from cell lysates using a modified Folch procedure (26). Radioactivity in aliquots of cell aqueous phase, lipid phase, and medium was quantitated by liquid scintillation counting. Calculation of substrate incorporation was based on the specific activity of labelled precursor added to the medium. Recovery of label typically was 90 ± 5%. Analysis of labelled lipids by thin-layer chromatography on Silica Gel G (Fisher Scientific, Ottawa, Canada) in chloroform/methanol/acetic acid/water (85:15:10:3.5) revealed that virtually all label from [^{14}C]ethanolamine was present as [^{14}C]phosphatidylethanolamine ([^{14}C]PtdEtn) and all [^{3}H]choline label as [^{3}H]phosphatidylcholine ([^{3}H]PtdCho) with or without agents present.

Pulse–chase experiments. In one type of pulse–chase experiment, preincubation conditions were identical to those described for pulse studies, except that either [^{14}C]ethanolamine (65 μM; 1.5 μCi) or [^{3}H]choline (7 μM; 3 μCi) was present in the culture medium. After 24 h, medium was removed and cells were washed twice with 2 mL MEM. Fresh chase medium (2 mL MEM) containing appropriate reagents and either 100 μM ethanolamine or 1 mM unlabelled choline was added. After 2 h incubation, chase medium was collected, and cells were harvested and processed as described previously for pulse studies. Similar experimental conditions have been used previously to evaluate catabolism of prelabelled phospholipids by measuring release of labelled head group to the culture medium (17,27–29). In other experiments to examine potential differences in cellular uptake of labeled precursors, cultures were pulsed with radiolabel for 1 h and chased for 30 min in the presence or absence of test agents.

PKC assay. Enzyme activity was measured in cytosolic and particulate cell fractions using a commercially available assay kit. Cultures were washed twice with 1 mL ice-cold phosphate buffered saline (pH 7.4) and incubated on ice for 5 min in 1 mL solubilization buffer (50 mM Tris-HCl, 0.5 mg digitonin/mL, 5 mM EGTA, 2 mM EDTA, 0.2 mM PMSF, 1 mM leupeptin, pH 7.5) to yield the cytosolic fraction (30). The particulate fraction was suspended in 1 mM lysis buffer (50 mM Tris-HCl, 250 mM sucrose, 5 mM EGTA, 2 mM EDTA, 0.2 mM PMSF, pH 7.5) and disrupted by sonication. Cell fractions were kept on ice and enzyme activity determined immediately, using a modified mixed micelle assay (31) following activation with β-TPA. PKC activity was measured as Ca^{2+}- and PtdSer-dependent incorporation of ^{32}P from [γ-^{32}P]ATP into substrate peptide (supplied in assay kit). Protein content ranged from 4–8 μg in a 75-μL incubation. Reaction was initiated with addition of [γ-^{32}P]ATP and, after 15 min incubation at 25°C in assay buffer (50 mM Tris-HCl, 1 mM calcium acetate, 8 mole% phosphatidylserine, 13 μM β-TPA, 75 μM substrate peptide, 2.5 μM DTT, 50 μM ATP, and 3.75 mM magnesium acetate, pH 7.5), was terminated with 100 μL of dilute acid (provided in assay kit). After mixing, 125 μL aliquots of reaction mixture were spotted on 6.25 cm^2 binding papers. Squares were washed twice for 10 min in 500 mL 5% acetic acid, transferred to scintillation vials, and counted. All values were corrected for background radioactivity (determined from reaction mixtures containing buffer instead of enzyme).

Statistical analysis. Results represent mean ± SEM. Statistical significance was determined by Student's *t*-test.

RESULTS

Effects of CLF and RA on EPG and choline phosphoglyceride synthesis and turnover. The effects of CLF and RA on

[^{14}C]ethanolamine incorporation into EPG by cultured human skin fibroblasts were examined during 15 min and 2 h incubations. Agent concentrations were based on preliminary dose–response experiments and previous investigations (16) such that possible synergism between the two agents could be assessed; accordingly, conditions were established to give relatively low levels of inhibition or activation (10–20%) by the agents alone, so that greater than additive effects potentially could be demonstrated. Figure 1A shows that CLF inhibited [^{14}C]ethanolamine incorporation into EPG during both 15-min and 2-h incubations, confirming earlier observations with other precursors of EPG (16). Inhibition was more pronounced during the 15-min incubation. RA also inhibited [^{14}C]ethanolamine incorporation into EPG, but the level of inhibition was similar at both times. Simultaneous addition of CLF and RA resulted in a level of inhibition at 15 min similar to that obtained with CLF alone. By contrast, the inhibition resulting from simultaneous exposure to both agents for 2 h was equivalent to the sum of the effects of each agent alone. Total cell-associated radioactivity was not altered by these agents, either alone or in combination, indicating that uptake of labelled ethanolamine was not a limiting factor.

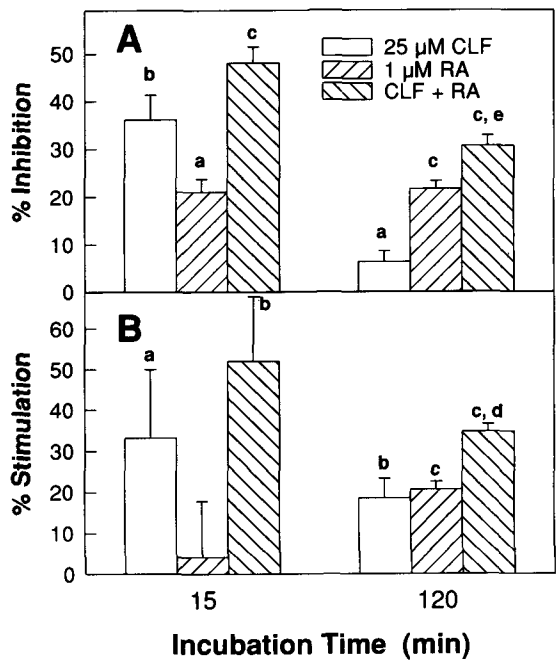


FIG. 1. Effects of clofibrate (CLF) and retinoic acid (RA) on [^{14}C]ethanolamine and [^{3}H]choline incorporation into phospholipids by cultured human skin fibroblasts. Cells were incubated for 15 or 120 min with either [1,2-^{14}C]ethanolamine (1.5 μCi, 67 μM; panel A) or ([methyl-^{3}H]choline, 3 μCi, 7 μM; panel B) and either 25 μM CLF, 1 μM RA, or both agents simultaneously. Control cells were incubated with equivalent volume of dimethylsulfoxide (DMSO) vehicle. Cells were harvested, lipids extracted, and radioactivity determined, as indicated in the Materials and Methods section. In panel A, data are expressed as percentage inhibition, where 0% inhibition (incorporation under control conditions) equals 48 ± 5 and 755 ± 55 pmol/mg protein for 15- and 120-min incubations, respectively. In panel B, results are shown as percentage stimulation, where 0% stimulation (control incorporation) equals 3.6 ± 0.3 and 152 ± 8 pmol/mg protein for 15- and 120-min incubations, respectively. Each value represents the mean ± SEM of $n = 4$–12. a, $P < 0.05$; b, $P < 0.005$, c, $P < 0.0005$, agent(s) vs. DMSO control; d, $P < 0.05$; e, $P < 0.005$, both agents together vs. each agent alone.

In distinct contrast, CLF stimulated [^{3}H]choline incorporation into PtdCho during both 15-min and 2-h incubations (Fig. 1B). Although RA was without effect during the shorter incubation time, it was significantly stimulatory at 2 h. When CLF and RA were added simultaneously, the stimulatory effect at 15 min was not different from that achieved with CLF alone. By contrast, simultaneous exposure to both agents for 2 h resulted in an additive stimulation of [^{3}H]choline incorporation into PtdCho. These agents, alone or in combination, did not alter total cell-associated radioactivity, again indicating that uptake of labelled choline was not a limiting factor.

To further confirm this latter point, effects of CLF and RA on incorporation of [^{3}H]choline from the cellular aqueous fraction into lipid-soluble components, independent of choline uptake or transport across the plasma membrane, were determined in 1-h pulse–30-min chase studies. Similar to the observations with the pulse experiments, CLF (25 μM) stimulated [^{3}H]choline incorporation into phospholipid (174 ± 12% of control, $n = 6$, $P < 0.005$). One μM RA was without effect (95 ± 17%, $n = 5$); however, 10 μM RA stimulated the incorporation of [^{3}H]choline into lipid-soluble components (212 ± 37%, $n = 6$, $P < 0.01$). Simultaneous exposure to both agents (25 μM CLF and 1 μM RA) resulted in a greater-than-additive stimulation of [^{3}H]choline incorporation into phospholipid.

The effects of CLF and RA on catabolism of EPG and choline phosphoglycerides and release of metabolites to the culture medium following long-term prelabelling were examined in 24-h pulse–2-h chase experiments. CLF stimulated release of [^{14}C]metabolites to the medium from [^{14}C]ethanolamine-labelled EPG. RA was without effect (Fig. 2). The effect of the two agents added simultaneously was not significantly different from that observed with CLF alone. Results were similar in experiments with 1 μM unlabelled choline instead of 100 μM ethanolamine in the chase medium (data not shown), suggesting that preferential uptake or recycling of ethanolamine and choline metabolites was not appreciable.

By contrast, CLF slightly inhibited the release of radioactive metabolites to the culture medium from [^{3}H]choline-labelled cells (Fig. 2). RA, at 1 μM, either alone or in combination with 25 μM clofibrate, did not significantly affect the release of label to the medium. However, inhibition of label release was evident with 10 μM RA [34.8 ± 2.5% inhibition, ($n = 6$), $P < 0.0005$].

Evaluation of protein kinase involvement in CLF- and RA-induced alterations in ethanolamine and choline phosphoglyceride metabolism. RA or CLF at concentrations and in combinations used to alter phospholipid synthesis and turnover had no appreciable effect on cellular PKC activities in either cytosolic or particulate fractions (Fig. 3). In contrast, 100 nM β-TPA caused translocation of PKC activity from the cytosolic to the particulate fraction and 2 μM β-TPA treat-

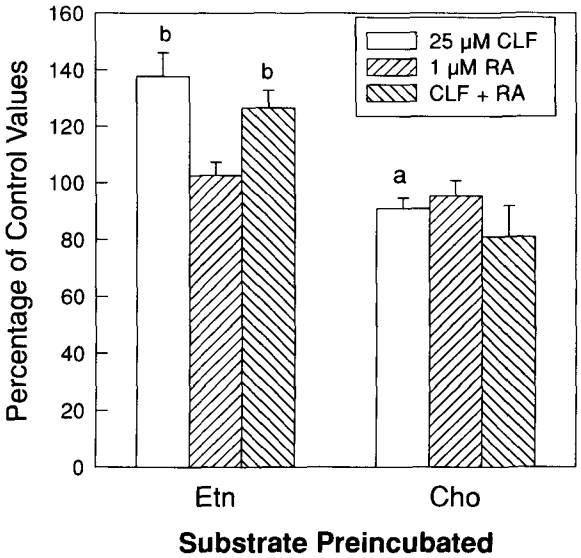


FIG. 2. Effects of CLF and RA on release of label from [^{14}C]ethanolamine (Etn) and [^{3}H]choline (Cho) to medium of cultured human skin fibroblasts. Cells were pulse-labelled for 24 h with either [1,2-^{14}C]ethanolamine (1.5 μCi, 67 μM) or [methyl ^{3}H]choline (3 μCi, 7 μM). Pulse medium was then removed and cells washed with fresh medium. Chase medium containing either 25 μM CLF, 1 μM RA, or both agents simultaneously, as well as 100 μM unlabelled ethanolamine or 1 mM choline, to dilute and trap released radioactive metabolites, was added and cells were incubated for 2 h. Cells were harvested, lipids were extracted, and radioactivity in the medium was determined as described in the Materials and Methods section. Data are expressed as percentage stimulation, where 0% stimulation (release from control cells incubated with DMSO vehicle) equals 4849 ± 312 pmol/mg protein for ethanolamine and 475 ± 76 for choline. Each value represents the mean ± SEM of $n = 6$. a, $P < 0.05$, b, $P < 0.005$ agent(s) vs. DMSO control. See Figure 1 for other abbreviations.

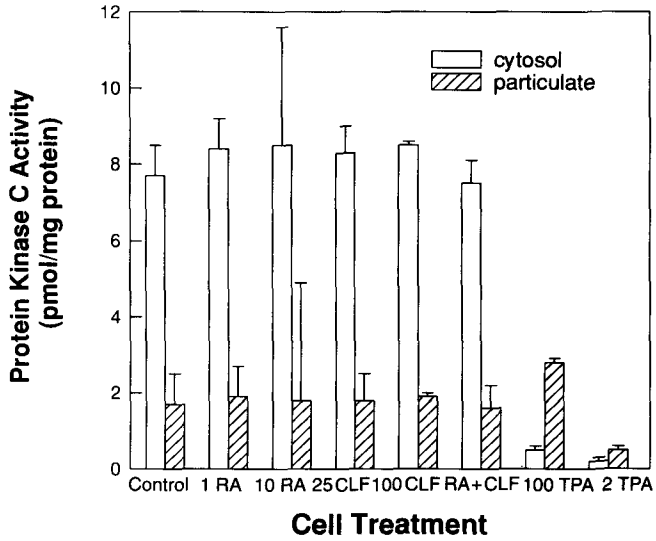


FIG. 3. Effects of CLF and RA on protein kinase C (PKC) activity in cultured human skin fibroblasts. Cells were incubated for 15 min with either RA (1 or 10 μM), CLF (25 or 100 μM), or both agents simultaneously (25 μM CLF and 1 μM RA). Control cells were incubated with an equivalent volume of DMSO. Cells also were treated with 100 nM β-TPA (β-12-*O*-tetradecanoylphorbol-13-acetate) for 15 min (100 TPA) or 2 μM β-TPA for 24 h (2 TPA). Cytosolic and particulate fractions were prepared and PKC activity determined, as described in the Materials and Methods section. Each value represents the mean ± SEM of $n = 2$–6 See Figure 1 for other abbreviations.

ment for 24 h gave appreciable downregulation of PKC activity in both fractions. Activation of PKC by exposure to 100 nM β-TPA for 15 min was without significant effect (8.1 ± 7.3% inhibition, $n = 8$) on [^{14}C]ethanolamine incorporation into EPG, whereas [^{3}H]choline incorporation into phospholipid was stimulated >3-fold, as shown for other cultured cells (17,32). The effects of two protein kinase inhibitors, H-7 and HA-1004 (33,34), on [^{14}C]ethanolamine and [^{3}H]choline incorporation into phospholipid, were compared in the absence and presence of CLF and RA (Fig. 4). Neither H-7 nor HA-1004 had any effect on basal [^{14}C]ethanolamine incorporation into EPG by control cultures, i.e., treated with DMSO vehicle during a 2-h incubation (Fig. 4A). CLF-induced inhibition of label incorporation was completely reversed by co-incubation with 100 μM H-7. By contrast, 100 μM HA-1004 was without effect. RA-treated cells also showed reduced [^{14}C]ethanolamine incorporation into phosphoglyceride relative to vehicle-treated controls. Co-incubation with either 100 μM H-7 or HA-1004 only partially countered the retinoid-induced inhibitory effect.

Incorporation of [^{3}H]choline into PtdCho by control cultures was unaffected by 100 μM HA-1004, but it was slightly increased ($P < 0.05$) by 100 μM H-7 (Fig. 4B). Co-incubation with 100 μM HA-1004 almost completely reversed the CLF-induced stimulation, whereas 100 μM H-7 had no effect. Neither H-7 nor HA-1004 significantly altered the stimulatory effect of RA. Although 100 μM H-7 was effective in inhibiting PKC *in vitro* (Fig. 5A, D), it had little effect on cellular PKC activity and subcellular distribution when incubated with intact cells (Fig. 5B, D). Similarly, treatment of intact cells with 100 μM HA-1004 did not alter PKC activity or distribution (Fig. 5B, E).

The effects of another protein kinase inhibitor, staurosporine, known to inhibit PKC and other kinases (35), were investigated. Staurosporine (5 μM) completely inhibited PKC activity *in vitro* (Fig. 5A, F) and was almost equally effective with intact cells (Fig. 5B, F). Effects of staurosporine on [^{14}C]ethanolamine incorporation into EPG were investigated in cultures treated with either vehicle, CLF or RA. Staurosporine (5 μM) inhibited [^{14}C]ethanolamine incorporation by control cultures treated with DMSO by 20.1 ± 1.3% ($n = 4$, $P < 0.0005$) during a 2-h incubation. Cells treated with 100 μM CLF showed a 27.5 ± 2.2% ($n = 9$) reduction in [^{14}C]ethanolamine incorporation into phospholipid relative to control. Co-incubation with staurosporine resulted in a level of inhibition (28.6 ± 7.3%, $n = 10$) similar to that observed with either compound alone. Cultures treated with 2 μM RA also exhibited decreased [^{14}C]ethanolamine incorporation (33.1 ± 1.7%, $n = 6$). Simultaneous exposure to the retinoid and staurosporine resulted in additive inhibition of [^{14}C]ethanolamine incorporation (47.2 ± 2.4%, $n = 6$). Another agent, *bis*-indolylmaleimide, has been reported to have

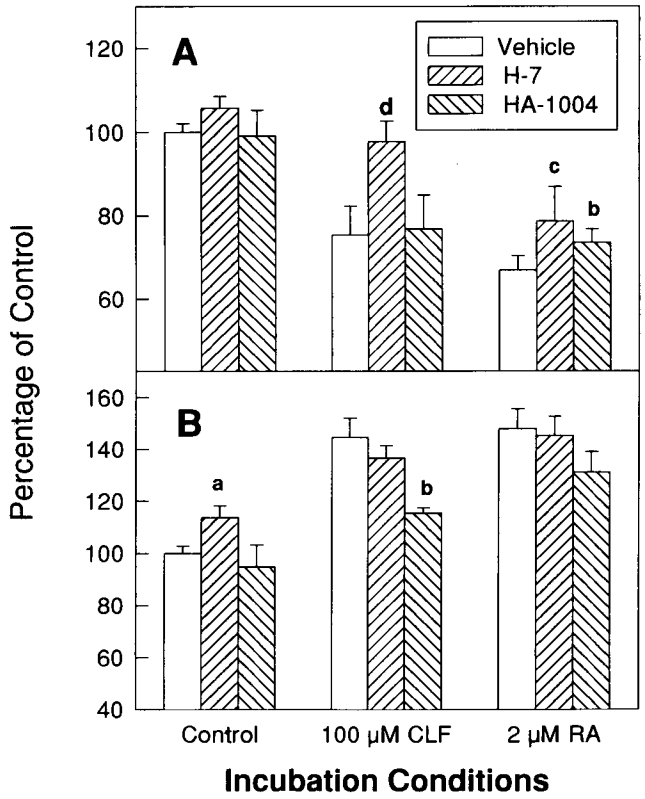


FIG. 4. Effects of protein kinase inhibitors on [^{14}C]ethanolamine and [^{3}H]choline incorporation into phospholipids by cultured human skin fibroblasts. Cells were incubated for 2 h with either [1,2-^{14}C]ethanolamine (1.5 μC, 67 μM; panel A) or [methyl-^{3}H]choline (3 μCi, 7 μM; panel B), and either 100 μM CLF, 2 μM RA, or an equivalent volume of DMSO (control) as indicated. Incubations also contained either 100 μM 1-(5-isoquinolinesulfonyl-2-methylpiperazine dihydrochloride H-7 (LC Laboratories, Woburn, MA), 100 μM *N*-(2′-guanidinoethyl)-5-isoquinolinesulfonamide (HA-1004; LC Laboratories), or an equivalent volume of water. Cells were harvested, lipids extracted, and radioactivity determined. Data are expressed as percentage of control (measured in presence of DMSO and water only), where control incorporation equals 327 ± 25 and 152 ± 8 pmol/mg protein in panels A and B, respectively. Each value represents the mean ± SEM of n = 4–12 for panel A and n = 4–6 for panel B. a, $P <$ 0.05 protein kinase inhibitor vs. DMSO control; b, $P < 0.05$; c, $P < 0.005$; d, $P < 0.0005$ protein kinase inhibitor and agent vs. agent alone. See Figure 1 for abbreviations.

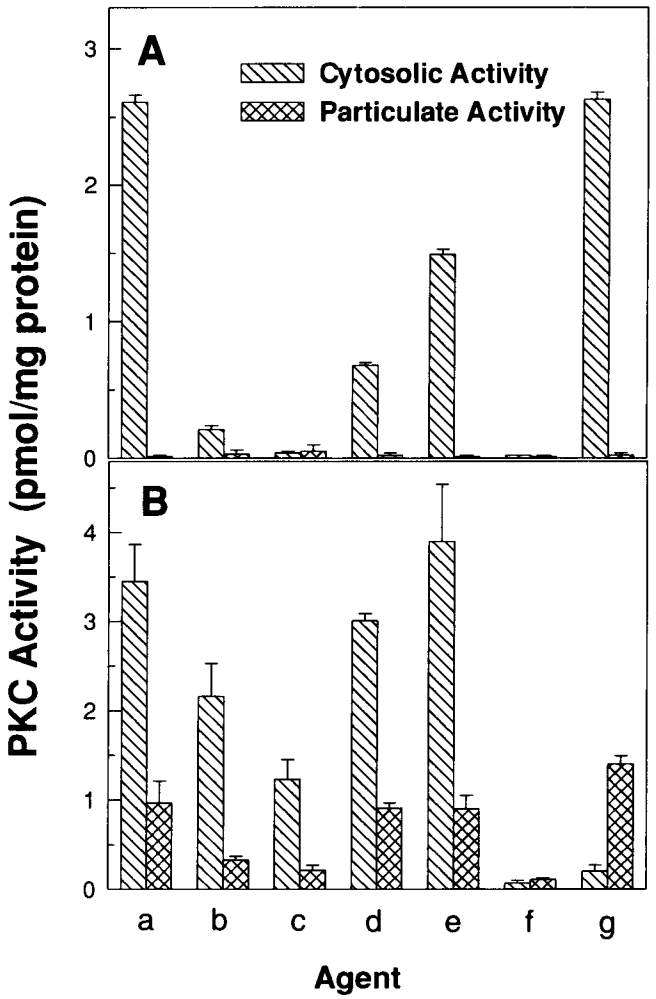


FIG. 5. Effects of various agents on activity of PKC in cytosolic and particulate fractions of fibroblast cells. In panel A, cytosolic and particulate fractions were isolated from fibroblasts as described in the Materials and Methods section and were incubated for 15 min with each agent added to the *in vitro* assay. In panel B, fibroblasts were incubated for 2 h with each agent before isolation of cytosolic and particulate fractions, and then PKC activity was assayed *in vitro* with optimal assay parameters. Conditions tested were: a, control with addition of the solvent carrier of agents to the cells or *in vitro* assay; b, 0.1 μM *bis*-indolylmaleimide; c, 1 μM *bis*-indolylmaleimide; d, 100 μM H-7; e, 100 μM HA-1004; f, 5 μM staurosporine; g, 0.1 μM β-TPA. Values are means ± SEM for n = 4. See Figure 3 for abbreviations and Figure 4 for company source.

high selectivity and potency for inhibition of PKC (36). One μM *bis*-indolylmaleimide effectively inhibited PKC *in vitro* (Fig. 5A, B, C) and reduced total cellular PKC in both cytosolic and particulate fractions of pretreated cells by at least 70% (Fig. 5B, C). In addition, 100 μM *bis*-indolylmaleimide completely inhibited phorbol ester-stimulated (β-TPA; Fig. 6B) incorporation of choline into phospholipid. Despite these effects, *bis*-indolylmaleimide had no effect on control, or CLF- and RA-altered, levels of ethanolamine (Fig. 6A) incorporation into phospholipids.

The inhibitory effect of CLF on [^{14}C]ethanolamine incorporation into EPG was examined following downregulation of PKC by β-TPA. Cultures treated with 2 μM β-TPA for 24 h showed higher [^{14}C]ethanolamine incorporation into phospholipid relative to vehicle-treated control cells (778 ± 29 vs. 568 ± 16 pmol/mg protein, respectively). Thus, phorbol ester- and vehicle-treated groups were compared after normalization to their respective controls. CLF (100 μM) inhibited [^{14}C]ethanolamine incorporation into EPG comparably in β-TPA- or vehicle-treated cells (39 ± 3% inhibition vs. 33 ± 3%, n = 6, respectively). At a lower concentration (25 μM) of the peroxisomal proliferator, a similar lack of a β-TPA effect on inhibition also was observed. Further, β-TPA-induced PKC downregulation did not alter RA-induced inhibition of [^{14}C]ethanolamine incorporation either at 1 μM (8.6 ± 3.9% vs. 11.9 ± 1.9%, n = 6 for β-TPA- and vehicle-treated cells, respectively) or 10 μM RA (72.6 ± 1.7% vs. 68.2 ± 1.6%, n = 6).

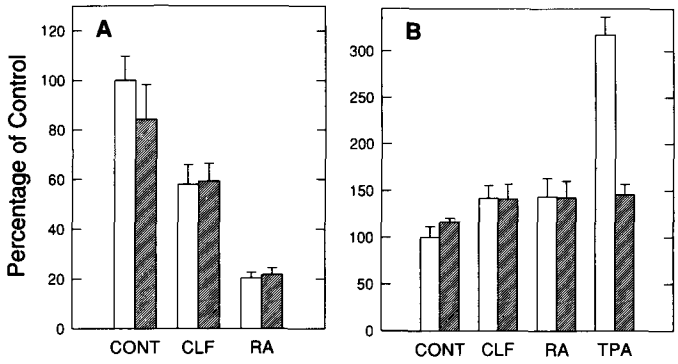


FIG. 6. Effects of *bis*-indolylmaleimide on CLF-, RA-, and phorbol ester induced alterations of choline and ethanolamine incorporation into lipids of fibroblast cells. In panel A, cells were incubated for 2 h with [1,2-^{14}C]ethanolamine (1.5 μCi, 67 μM) and in panel B [methyl-^{3}H]choline (3 μCi, 7 μM). Incubations also included 100 μM CLF, 2 μM RA, or 100 nM β-TPA, without (white bar) or with (striped bar) 1 μM *bis*-indolylmaleimide present. Control cells were incubated with equivalent volume of vehicle. Values are means ± SEM for n = 4–8. CONT is control. See Figures 1 and 3 for other abbreviations.

Incorporation of [^{14}C]ethanolamine was inhibited by forskolin (16.1% at 0.1 μM and 33.0% at 1 μM forskolin), whereas dideoxyforskolin (0.01–10 μM) had no effect. In combination with CLF or RA, forskolin did not alter the effects of either agent. Effects of forskolin alone on [^{3}H]choline incorporation were indistinguishable from those of dideoxyforskolin, and in combination with the other agents, forskolin did not alter effects of either CLF or RA.

DISCUSSION

Influences of peroxisomal proliferators, such as CLF, and of retinoids, such as RA, on cellular metabolism and physiology are numerous and varied (9,37–39). Some studies indicate similarities between these classes of agents and the potential for interaction at the level of common or shared receptor and response elements (13–15). Based on earlier observations that CLF somewhat specifically alters EPG metabolism in cultured fibroblasts (16), we have investigated relationships between CLF and RA in modifying membrane lipid turnover. As both similarities and differences in the effects acid of the two agents on synthesis and catabolism of ethanolamine and choline phosphoglycerides were observed, we explored potential points of metabolic regulation of these effects.

From these studies, it is apparent that ethanolamine and choline phosphoglyceride metabolism in cultured skin fibroblast cells involves different levels of regulation by CLF and RA. Although the effect of CLF on both phospholipid classes is rapid, occurring within 15 min, inhibition of ethanolamine incorporation into EPG by CLF diminishes with time; in contrast, stimulation of choline incorporation by this agent remains elevated after two hours of incubation. This observation is consistent with earlier findings (16) that ethanolamine phospholipid synthesis in skin fibroblasts undergoes a time-dependent release from CLF-induced inhibition. The longer (18–24 h) recovery period in that earlier study probably relates to the higher concentration (100 μM) of CLF and use of a different precursor to monitor phospholipid turnover. Metabolic inactivation of CLF is unlikely to account for reversal of inhibition of ethanolamine incorporation, as it is not observed in experiments with choline. Rather, our findings suggest differences in the mode of action of CLF with regard to the two phospholipid classes. A similar differential effect was observed with RA. The inhibitory effect of the retinoid on ethanolamine incorporation into EPG is rapid and sustained, but RA-induced stimulation of choline incorporation is slower.

Previous studies describing effects of peroxisomal proliferators and retinoids on lipid metabolism have focused on β-oxidation of fatty acids (40–42) and other aspects of lipid metabolism (37,38,43–45). Although each agent independently activates genes involved in fatty acid β-oxidation, simultaneous exposure to both activators results in synergistic induction of gene expression (14). Our findings indicate that the effects of CLF and RA on ethanolamine and choline incorporation into phosphoglycerides generally are additive rather than synergistic, supporting distinct, noninteracting modes of action for each agent rather than a convergence of regulatory pathways through retinoid receptors or PPAR.

CLF- and RA-induced alterations in the incorporation of ethanolamine and choline into phospholipid potentially could reflect effects of these agents on one or more metabolic processes, including uptake/transport of the exogenous precursor, biosynthesis involving several enzymes and alternative pathways (19,44) or degradation by lipases, including phospholipases C (PLC) and D (PLD). Uptake and transport of ethanolamine and choline seem unlikely targets for regulation in the present study, as neither CLF nor RA had any effect on total cell-associated radioactivity. Further, similar effects of these agents were observed when cells were prelabeled with base precursors in short-term pulse–chase experiments (to eliminate potential effects on uptake and transport). On the other hand, phosphoglyceride degradation appears to be involved as a potential regulatory target at least in the case of CLF. Long-term pulse–chase experiments with ethanolamine (to label the EPG pool, followed by chase in the presence of CLF to determine effects on degradation of phospholipid) show CLF increases label release to the culture medium, indicating stimulation of EPG degradation. In contrast, degradation of prelabeled PtdCho is inhibited by this agent, in agreement with *in vivo* observations in rodents (44). Several agonists have been shown to influence degradation of PtdCho through their action on PLC or PLD (46–48). No effects on degradation of either phospholipid were observed with RA.

Based on previous reports of interactions between PKC activity and CLF and retinoids (20–22), we examined the possibility that PKC might be mediating some or all of the effects of these agents on differential turnover of PtdCho and PtdEtn. H-7 is widely used as an inhibitor of protein kinases (33,34); although potent, it is relatively nonspecific, with similar IC_{50} values for PKC, PKA, and PKG (protein kinases A and G)

(3–6 μM). HA-1004, although a good inhibitor of PKA and PKG, is tenfold less effective as an inhibitor of PKC. By comparing relative abilities of these protein kinase inhibitors to prevent CLF- or RA-induced alterations in ethanolamine or choline phospholipid turnover, we anticipated evidence for which of these protein kinases, if any, are likely mediators of the observed responses. CLF-induced inhibition of ethanolamine incorporation into EPG was prevented completely upon incubation with H-7, but not HA-1004, suggesting PKC involvement in this process. By contrast, both H-7 and HA-1004 partially countered the inhibitory effect of RA on ethanolamine incorporation, suggesting that at least part of this retinoid effect may be mediated by kinases. The stimulatory effect of CLF on choline incorporation into PtdCho was partially reversed by HA-1004, consistent with at least part of the drug's action being mediated by other kinases. Neither protein kinase inhibitor prevented the RA-induced stimulation of choline incorporation, suggesting that kinases were not involved in this process. Lack of effect of RA on choline incorporation at 15 min further supported involvement of a protein kinase-independent mechanism. Thus, our observations implicated kinase-mediated signal transduction pathways in the regulation of some, but not all, aspects of ethanolamine and choline phospholipid turnover initiated by CLF and RA.

Potential involvement of PKC in CLF-induced inhibition of EPG turnover was more extensively examined. Several approaches were taken, as none of the commonly used methods to assess PKC involvement is without limitations. Additional evidence argues against a role for classical PKC isoforms: (i) *bis*-indolylmaleimide, a highly selective PKC inhibitor (36), did not reverse the inhibitory effect of clofibrate on ethanolamine incorporation into EPG; this agent effectively inhibited fibroblast PKC activity *in vitro,* although even at the highest (1 μM) concentration tested with intact cells, as much as 30% of PKC activity remained; (ii) TPA-induced downregulation of PKC, under conditions that eliminated >90% of cytosolic PKC activity, did not prevent CLF-induced inhibition of ethanolamine incorporation; however, substantial membrane activity remained; (iii) TPA-induced activation of PKC did not mimic the inhibitory effects of clofibrate; and (iv) CLF did not activate PKC in either cytosolic or particulate cellular fractions. Accordingly, our data indicate that classical, TPA-sensitive isoforms of PKC do not mediate the effects of CLF and RA on phospholipid turnover. However, given the residual PKC activity that remains following TPA-induced downregulation or *bis*-indolylmaleimide treatment, we cannot exclude the possibility that isoforms in the atypical class of PKC (23,49), some of which are found in fibroblast and other skin cells (50,51), may be involved.

Results with the nonspecific kinase inhibitor, staurosporine, differed from those obtained with H-7 and *bis*-indolylmaleimide. Although basal ethanolamine incorporation decreased in response to staurosporine treatment, this inhibition could involve more than inhibition of PKC. Micromolar concentrations of staurosporine have been shown to elicit effects, such as direct activation of PLD, independent of PKC inhibition (52). Activation of phospholipases, possibly PLD, may be one means whereby CLF alters ethanolamine incorporation into phospholipid, but direct evidence remains to be determined. Studies with forskolin suggest that activation of PKA may inhibit EPG synthesis but, in combination with CLF and RA, do not support involvement of PKA as an explanation for the action of these two agents.

In summary, our study supports differential regulation of ethanolamine and choline phosphoglyceride turnover by clofibrate and retinoic acid. Ethanolamine incorporation into phospholipid is inhibited, whereas PtdCho turnover is stimulated by these agents. Alterations in both biosynthesis and degradation are involved. Mechanisms underlying CLF- and RA-induced alterations in ethanolamine and choline phospholipid turnover differ and appear not to involve TPA-sensitive PKC isoforms.

ACKNOWLEDGMENTS

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The High Content of Monoene Fatty Acids in the Lipids of Some Midwater Fishes: Family Myctophidae

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The total lipids of eleven species of Myctophids caught at depths between 20 and 700 m in the northern Pacific Ocean were analyzed using silicic acid column chromatography (lipid classes) and capillary gas chromatography (fatty acid and fatty alcohol composition). The major components in the lipid classes were triacylglycerols or wax esters; triacylglycerols were the dominant acyl neutral lipids (68.1–96.1%) in eight species, and wax esters were found as the dominant lipid (85.5–87.9%) in three species. The major fatty acids and alcohols contained in the wax esters of the three fishes were 18:1n-9, 20:1n-9, 20:1n-11, and 22:1n-11 for fatty acids, and 16:0, 18:1, 20:1, and 22:1 for fatty alcohols. Fatty acids in the triacylglycerols ranging from C_{14} to C_{22} were predominantly of even chain length. The major components were 16:0, 16:1n-7, 18:1n-9, 20:1n-11, 22:1n-11, 20:5n-3 (icosapentaenoic acid), and 22:6n-3 (docosahexaenoic acid). In both the triacylglycerols and the wax esters, the major fatty components were monoenoic acids and alcohols. It is suggested from the lipid chemistry of the Myctophids that they may prey on the same organisms as the certain pelagic fishes such as saury and herring, because the large quantities of monoenoic fatty acids are similar to those of saury, herring, and sprats whose lipids originate from their prey organisms such as zooplanktons which are rich in monoenoic wax esters.
Lipids 31, 757–763 (1996).

It has been reported that triacylglycerols are usually contained in the lipids of pelagic surface fishes as deposit lipids; however, deep-sea teleost fishes often have considerable amounts of wax esters as a substitute for glycerides (1–3). Fatty acids and other lipid components of deep-sea fish have been examined by Nevenzel and Menon (4) and Nevenzel (5), Buisson *et al.* (6), Takagi *et al.* (7), and Bakes *et al.* (8).

The deep-sea fish, family Myctophidae, is the most widespread family of the mesopelagic deep-sea fishes in the world's oceans. Myctophids are known to be important prey items of various marine animals, and the lipid of the Myctophids is an important energy source for higher predatory fishes (9). For example, Nevenzel *et al.* (10) reported the fatty acid and alcohol compositions of wax esters from the lipids of some Myctophid species, and Kayama and Ikeda (11) reported the wax esters in some deep-sea fishes Myctophidae.

The present paper deals with the analyses of lipids of eleven species of Myctophids caught at different depths, which are common and numerous species in the transition zone between the Oyashio (the cold current off the eastern Japanese coast) and the Kuroshio (the warm current running along the southern coast of Japan) in the northern Pacific Ocean. In addition, the lipid classes and the fatty acid and alcohol compositions of the individual lipid classes are compared with those of other marine pelagic fish species already reported to discuss the relationship between the lipids of Myctophids and their feeding habits.

MATERIALS AND METHODS

Materials. All specimens of Myctophids were caught by trawlers belonging to the Japan Marine Fishery Resource Research Center between latitudes 39 to 42°N and longitudes 143 to 150°E in the northern Pacific Ocean in August 1994. The seawater temperatures were between about 4°C (at depths 350–700 m) and 20°C (at depth 20–50 m).

All samples were frozen immediately, and kept frozen at –40°C for three weeks until extracted. Identifications generally were made by qualified experts, as acknowledged later.

Lipid extraction and the analysis of lipid classes. The total lipids from all specimens of Myctophids were obtained by extraction into chloroform/methanol (2:1, vol/vol) according to the Folch procedure (12). The crude total lipids were separated into classes on silicic acid columns, and the quantitative analysis of the constituents of the lipids was carried out by gravimetric analysis of column chromatography. The glass columns (ϕ 30 mm × 400 mm) were prepared with 50 g of silicic acid (Kieselgel 60, 70–230 mesh; Merck & Co. Ltd., Darmstadt, Germany) suspended in dichloromethane/*n*-hexane (2:3, vol/vol). The sample (300 mg) of total lipids in 3 mL of the same solvent was placed on top of the packing just as it was draining dry. The sample was followed with a few small portions (3–5 mL) of the same solvent and then additional solvent in a total amount of 300 mL. The eluate was collected as the wax ester fraction.

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Abbreviations: DHA, docosahexaenoic acid; EPA, icosapentaenoic acid; FAME, fatty acid methyl ester; GLC, gas–liquid chromatography; NMR, nuclear magnetic resonance; PUFA, polyunsaturated fatty acids, RRT, relative retention time.

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Dichloromethane (300 mL) was then passed through the column, and the triacylglycerol fraction was collected. This was followed with dichloro-methane/ether (35:1, vol/vol) eluting the diglycerides, dichloromethane/ether (9:1, vol/vol) eluting the sterols, dichloromethane/methanol (10:1, vol/vol) eluting the free fatty acids, and dichloromethane/methanol (1:5, vol/vol) and dichloromethane/methanol (1:20,vol/vol) eluting the phospholipids.

Individual lipids separated from each lipid class were identified with authentic samples by thin-layer chromatography (Kieselgel 60, thickness of 0.25 mm for analysis; Merck & Co. Ltd.), nuclear magnetic resonance (NMR), and mass spectrometry. All sample lipids were dried under argon at room temperature and stored at –40°C.

The preparation of methyl esters and gas–liquid chromatography (GLC). The individual components of triacylglycerols were converted to fatty acid methyl esters (FAME) by direct transesterification with boiling methanol containing 1% of concentrated hydrochloric acid under reflux for 1.5 h under argon atmosphere. The methyl esters thus obtained were purified by column chromatography with silica gel by elution with a mixture of dichloromethane and ether (10:1, vol/vol).

The wax esters fractionated also were converted to FAME and fatty alcohols by the same procedure, and the reaction mixture of the wax esters was separated by silicic column chromatography with a elution of the mixture of dichloromethane and ether (35:1, vol/vol), and then the FAME were purified, and the residual wax alcohols were purified with the elution by the similar solvents (dichloro-methane/ether, 9:1, vol/vol).

Analysis of FAME and fatty alcohols contained in the wax esters were performed on an HP 5890 series II gas chromatograph (Hewlett-Packard Co., Yokogawa Electric Corporation, Tokyo, Japan) equipped with an omegawax-320 fused silica capillary column (30 m × 0.32 mm i. d., 0.25 μm film; Supelco Japan Co. Ltd., Tokyo, Japan). The temperatures of the injector and the column were held at 250 and 190°C, respectively, and the split ratio was 1:100. Helium was used as the carrier gas at a constant inlet rate of 40 mL/min.

Quantitative analyses were performed on the capillary columns by means of a Shimadzu Model C-R5A (Shimadzu Seisakusho Co. Ltd., Kyoto, Japan) electronic integrator.

Peak identification. FAME and wax alcohols were identified using: (i) marine lipid methyl esters as standards (omegawax test mixture No. 4-8476; Supelco Japan Ltd.), (ii) semilogarithmic plot of relative retention time (RRT) against carbon chain lengths of the fatty acids of fish oil and fitting the logarithm of RRT of the fatty acids into these plots, comparison of the equivalent chain length values according to the method of Ackman (13), and (iii) comparison of the determination of the data by gas chromatograph mass spectroscopy (JMS-DX303; JEOL Co. Ltd., Tokyo, Japan) attached to the capillary column. The injector and separation temperature were 250 and 190°C, and the ionization voltage was 70 eV, respectively.

NMR spectrometry. Spectra were recorded on a GSX-270 NMR spectrometer (JEOL Co. Ltd.) in the pulsed Fourier transform mode at 270 MHz in deuterochloroform solution using tetramethylsilane as an internal standard.

Statistical analyses. More than three replications were made of the samples for all treatments. The significant differences of means for the data were inspected by Student's *t*-test at the level of $P < 0.05$.

RESULTS AND DISCUSSION

All specimens were caught at depths between 20 and 700 m in the northern Pacific Ocean between latitudes 39 to 42°N and longitudes 143 to 150°E in August 1994. The majority of these species show diel vertical migration because peak catch of samples occurred in the near-surface waters (20–200 m) at night and in the mesopelagic zone (400–700 m) during the daytime, with the exception of the three stationary species (*Lampanyctus jordani, L. regalis*, and *Stenobrachius nannochir*) (Table 1).

TABLE 1
The Average Lipid Content of the Fishes in Mycotophidae with Relevant Data[a]

Name	Catching depth (m)		Length (cm)	Weight (g)	Lipids (%)[b]
	Day	Night			
First group					
Notoscopelus japonicus	300–500	50–200	13.9 ± 0.6	36.3 ± 5.1	26.0 ± 3.8
Symbolophorus californiensis	400–500	20–100	10.7 ± 0.4	17.5 ± 3.3	25.4 ± 4.1
Lampadena luminosa	400–700	—	18.5 ± 1.6	103.5 ± 37.0	20.0 ± 1.4
Second group					
Protomyctophum thompsoni	300–500	—	4.6 ± 0.1	1.6 ± 0.1	12.4 ± 1.3
Ceratoscopelus warmingi	400–500	50–100	8.0 ± 0.4	7.6 ± 1.0	18.6 ± 3.1
Stenobrachius leucopsarus	400–500	20–100	8.5 ± 0.8	8.2 ± 2.3	15.7 ± 0.9
S. nannochir	500–700	500–700	10.4 ± 1.5	12.9 ± 6.5	13.4 ± 2.4
Lampanyctus regalis	500–700	500–700	18.5 ± 1.2	65.7 ± 10.9	12.0 ± 2.5
Third group					
Diaphus theta	300–600	50–200	9.2 ± 0.4	11.3 ± 1.1	3.5 ± 1.0
D. gigus	400–600	20–150	9.0 ± 0.7	11.5 ± 2.1	5.1 ± 2.0
L. jordani	400–700	500–600	11.3 ± 0.5	17.8 ± 3.8	6.8 ± 3.0

[a]Results are expressed as mean ± SD for five samples.
[b]Results of lipid contents are expressed as weight percentage of wet tissues.

Lipid contents in the respective species. The lipid content of the whole bodies of the sampled Myctophids with their weights and standard lengths are shown in Table 1, and the total lipid content based on the fresh tissue varied between species and ranged between 3.5 to 26.0%. The examined Myctophids were divided into three types according to the lipid contents ($P < 0.05$); the first group of high lipid content (20.0–26.0%, *Notoscopelus japonicus*, *Symbolophorus californiensis*, and *Lampadena luminosa*); the second group of medium lipid content (12.0–18.6%, *Protomyctophum thompsoni*, *S. leucopsarus*, *Ceratoscopelus warmingi*, *S. nannochir*, and *L. regalis*); the third group of the low lipid content (3.5–6.8%, *Diaphus theta*, *D. gigus*, and *L. jordani*).

Lipid classes (analysis of lipid composition of total lipids). The total lipids were fractionated into various classes as neutral lipids (wax esters, triacylglycerols, and sterols) and polar lipids (phospholipids) by silicic column chromatography, and the lipid compositions are given in Table 2. The lipids of all species examined generally contained a large amount of neutral lipids (85.3–98.6%) with only small quantities of polar lipids (1.4–13.2%).

All of the three fatty fish species belonging to the first group have significantly high ratios of triacylglycerols (93.7–96.1%) in total lipids, and the three lean species of the third group have high ratios of phospholipids (7.3–13.2%) ($P < 0.05$). The total lipid of eight species of Myctophids were mainly comprised of glyceride derivatives such as triacylglycerols (the eight species in the first, second, and third groups) and phospholipids (the three species in the third group). Triacylglycerols (85.7 ± 9.3%) were the major component among their lipid classes; sterols and phospholipids were found as minor components, and wax esters were only slightly detected (0.4–2.0%).

The three species (*S. leucopsarus*, *S. nannochir*, and *L. regalis*) belonging to the second group have wax ester as the major component with a small amount of glycerides derivatives and sterols being found (10). Low levels of sterols (5.4 ± 5.1%) in the free and combined state were encountered in all samples, and they were mostly nonesterified. The sterol distributions were very simple, with only cholesterol accounting for >99% of the total sterols identified with the authentic cholesterol by the measurement of NMR and GLC equipped with a fused silica capillary column (HP-5, 30 m × 0.32 mm i.d.; 0.25 μm film, Hewlett-Packard Co., Yokogawa Electric Corporation). Hydrocarbons were hardly detected in any specimen examined.

In the three Myctophid species, which had similar lipid compositions containing *ca.* 90% (85.5–87.9%) wax esters, *S. nannochir* and *L. regalis* showed no diel vertical migration patterns being caught day and night between 400 and 700 m, while *S. leucopsarus* was caught at two different depths by day (400–500 m) and by night (20–100 m). The seven Myctophid species, which had high triacylglycerol contents, showed clear vertical migrations being caught at different depths by day and by night with the exception of *L. jordani* (Tables 1 and 2). Therefore, there may be some relationship between the Myctophids containing the triacylglycerols as the dominant lipid and the behavior of diel vertical migration.

Fatty acid and fatty alcohol compositions of wax esters, major components for three species of the Myctophids. Fatty acids and alcohols of the wax esters in the three Myctophid species, *S. leucopsarus*, *S. nannochir*, and *L. regalis*, are shown in Table 3. The major components in the wax esters in all species were 16:1 (2.5–10.0%), 18:1 (31.0–37.5%), 20:1 (14.1–34.6%), and 22:1 (11.5–16.2%) for fatty acids, and 16:0 (4.3–35.7%), 18:1 (4.5–8.4%), 20:1 (9.8–22.7%), 22:1 (24.2–52.0%), and 24:1 (3.2–6.1%) for fatty alcohols. The total amounts of these major components reached 65.4–87.7 and 48.3–87.9% of the total fats for the acids and alcohols, respectively. Various kinds of fatty acids were found because more than 25 peaks were detected at a level of 0.3% or more of the total fatty acids; however, in the case of the alcohols, only ten kinds of the fatty alcohols were simply detected at the same level of the total fatty alcohols (Table 3). The alcohols were characterized by a predominance of 16:0, 18:1, 20:1, and 22:1, and the respective amounts of the other fatty

TABLE 2
The Lipid Classes of the Eleven Species in Myctophidae (%)[a]

	Wax esters	Triacylglycerols	Sterols	Phospholipids
First group				
Notoscopelus japonicus	0.5 ± 0.2	93.7 ± 0.4	3.0 ± 1.0	3.2 ± 1.2
Symbolophorus californiensis	0.6 ± 0.5	94.3 ± 0.8	2.0 ± 0.6	3.1 ± 0.5
Lampadena luminosa	1.0 ± 0.5	96.1 ± 2.1	1.3 ± 1.6	1.4 ± 0.6
Second group				
Protomyctophum thompsoni	1.2 ± 0.4	88.2 ± 0.8	5.3 ± 0.8	5.0 ± 1.0
Ceratoscopelus warmingi	0.4 ± 0.2	81.5 ± 6.5	8.9 ± 4.4[b]	9.2 ± 3.0
Stenobrachius leucopsarus	87.9 ± 1.0	6.9 ± 0.4	2.1 ± 0.6	2.7 ± 0.4
S. nannochir	87.9 ± 1.3	7.8 ± 1.6	1.7 ± 0.4	3.2 ± 0.6
Lampanyctus regalis	85.5 ± 4.8	10.4 ± 6.0	1.9 ± 0.4	2.1 ± 1.1
Third group				
Diaphus theta	0.6 ± 0.3	68.1 ± 25.7	18.1 ± 22.6[b]	13.2 ± 4.6
D. gigus	2.0 ± 1.1	80.8 ± 5.3	6.0 ± 1.1	11.1 ± 5.1
L. jordani	1.4 ± 0.2	83.0 ± 8.8	10.0 ± 10.3[b]	7.3 ± 2.9

[a]Results are expressed as weight percentage of total lipids. Data are mean ± SD for three samples.
[b]Contain small amounts of free fatty acids and diglycerides.

TABLE 3
The Fatty Acid and Alcohol Compositions in the Wax Esters of the Three Species in Myctophidae (%)[a]

	Stenobrachius leucopsarus		*S. nannochir*		*Lampanyctus regalis*	
	Acid	Alcohol	Acid	Alcohol	Acid	Alcohol
Total saturated	3.4	45.2	2.5	31.7	2.4	6.9
14:0	1.1 ± 1.2	5.9 ± 0.9	0.9 ± 0.1	7.9 ± 1.8	1.2 ± 0.1	0.9 ± 1.1
16:0	2.0 ± 0.3	35.7 ± 16.6	1.6 ± 1.1	21.6 ± 2.0	0.9 ± 0.4	4.3 ± 1.3
18:0	0.3 ± 0.2	3.3 ± 1.7	trace	1.9 ± 0.3	0.4 ± 0.2	1.2 ± 0.3
20:0		0.3 ± 0.0		0.3 ± 0.0		0.5 ± 0.0
Total monoenoic	65.4	48.9	84.3	62.6	87.7	89.3
16:1n-7	2.0 ± 0.3	0.9 ± 0.2	9.5 ± 3.8	0.7 ± 0.1	5.6 ± 0.4	2.0 ± 0.7
16:1n-5	0.5 ± 0.1	2.3 ± 0.5	0.5 ± 0.3	1.5 ± 0.2	trace	trace
18:1n-9	36.4 ± 10.1	6.0 ± 3.7	30.4 ± 0.2	2.8 ± 0.0	29.3 ± 2.9	4.7 ± 0.7
18:1n-7	1.1 ± 0.7	1.8 ± 1.1	1.2 ± 0.4	0.9 ± 0.1	1.7 ± 1.9	1.4 ± 0.3
18:1n-5	trace	0.6 ± 0.0	trace	0.8 ± 0.0	0.4 ± 0.2	0.4 ± 0.1
20:1n-11	13.7 ± 9.6[b]	6.3 ± 5.1	26.5 ± 3.1[b]	11.7 ± 1.5	23.6 ± 6.6	22.1 ± 1.3[b]
20:1n-9		3.1 ± 1.7		3.2 ± 0.3	10.7 ± 1.9	
20:1n-7	0.4 ± 0.1	0.4 ± 0.2	trace	0.5 ± 0.1	0.3 ± 0.3	0.6 ± 0.3
22:1n-11	10.6 ± 9.2	23.1 ± 16.0[c]	14.8 ± 5.2	35.7 ± 3.1[c]	15.3 ± 2.4	51.0 ± 3.9[c]
22:1n-9	0.5 ± 0.4		1.2 ± 0.1		0.4 ± 0.6	
22:1n-7		1.1 ± 0.5		1.1 ± 0.3		1.0 ± 0.8
24:1n-9	0.4 ± 0.4	3.2 ± 1.2[d]	0.4 ± 0.2	4.4 ± 0.2[d]	0.5 ± 0.3	6.1 ± 0.8[d]
Total polyenoic	18.4	0.3	8.1	0.4	6.9	0.5
16:2n-6	2.0 ± 0.3		1.6 ± 1.1		0.9 ± 0.5	
18:2n-6	2.6 ± 1.2		1.7 ± 0.4		1.8 ± 0.2	
20:2n-6	trace	0.3 ± 0.2	0.3 ± 0.1	0.4 ± 0.0	0.4 ± 0.2	0.5 ± 0.3
20:4n-6	0.4 ± 0.3		trace		trace	
18:3n-3	1.2 ± 0.8		0.6 ± 0.2		0.4 ± 0.1	
18:4n-3	1.3 ± 0.8		0.4 ± 0.2		0.4 ± 0.0	
20:3n-3	trace		trace		trace	
20:4n-3	0.6 ± 0.3		0.5 ± 0.1		0.6 ± 0.4	
20:5n-3	4.7 ± 3.7		1.2 ± 0.1		1.2 ± 1.2	
22:2n-3	0.5 ± 0.4		trace		trace	
22:5n-3	0.4 ± 0.3		0.3 ± 0.1		0.3 ± 0.3	
22:6n-3	4.7 ± 3.4		1.6 ± 0.2		1.0 ± 0.6	

[a]Results are expressed as weight percentage of total fatty acids. Data are mean ± SD for three samples, "trace" denotes <0.3%. [b]Mixture of 20:1n-9 and 20:1n-11. [c]Mixture of 22:1n-9 and 22:1n-11. [d]Mixture of 24:1n-9 and 24:1n-11.

alcohols were less than 0.1% or not detected (Table 3). The ratios of the total monoenoic fatty alcohols (48.3–87.9%) in the wax alcohols of the three species were very high and similar to those of other deep-sea fish species containing wax esters as a major component, and the same trend has been noted in the reports about the lipids of some other deep-sea fishes (14–17).

Although various kinds of fatty acids were observed in the wax esters of the Myctophids examined and were similar to those in general of other marine fish species, the markedly high levels of total monoenoic acids being found (65.4–87.7%) differ from other general marine fish species. In particular, the monoene fatty acids, 18:1 to 24:1 inclusive, made up 62.9–82.1% of the total acids similar to the case of the monoene alcohols (18:1, 20:1, 22:1, and 24:1) in their wax esters, because the total ratios of the alcohols were 45.1–85.9% of the total fatty alcohols. Sargent *et al.* (18) reported that the relatively large amount of monoenes in some midwater fishes and crustaceans was caused by high levels of wax esters. In accordance with Sargent *et al.* (18), our results suggest that Myctophids rich in wax esters have high proportions of monoenes in both the acid and the alcohol portion of the wax esters.

Fatty acid composition of triacylglycerols, major components for eight species of the Myctophids which vertically migrate except L. jordani. The mean fatty acid composition of the triacylglycerols as the major component in the lipids of eight species of Myctophids detected by GLC are compared in Table 4. Various kinds of fatty acids in the triacylglycerols of the eight species were detected (at least 25 peaks, Table 4) similar to the case of wax esters of the three species (Table 3). Even numbers of carbon atoms, ranging from C_{14} to C_{22}, were abundant as compared with odd numbers, 15:0 and 17:0, whose amounts were only in small quantities (<1%). The major saturated fatty acids of all species were 16:0 (10.3–23.0%) and 18:0 (2.0–7.5%). The major monounsaturated fatty acids were 16:1n-7 (3.3–7.3%), 18:1 (18.2–35.3%), 20:1 (3.3–13.3%), and 22:1 (1.5–16.4%), with 20:5n-3 (icosapentaenoic acid, EPA) (2.2-8.2%) and 22:6n-3 (docosahexaenoic acid, DHA) (3.3–8.6%) being the major

TABLE 4
The Fatty Acid Composition in the Triacylglycerols of the Eight Species in Myctophidae (%)[a]

	N. japonicus	*S. cariforniensis*	*L. luminosa*	*P. thompsoni*	*C. warmingi*	*D. theta*	*D. gigus*	*L. jordani*
Total saturated	21.8	21.9	17.8	22.0	33.8	32.0	27.3	20.6
14:0	1.5 ± 0.2	2.4 ± 0.5	2.3 ± 0.2	4.8 ± 0.6	2.6 ± 0.1	3.4 ± 0.1	3.6 ± 0.8	4.2 ± 1.2
15:0	trace	trace	trace	0.4 ± 0.0	0.3 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	trace
16:0	15.2 ± 1.3	14.1 ± 2.0	10.3 ± 1.5	14.3 ± 0.2	23.0 ± 0.9	21.2 ± 1.2	16.5 ± 1.1	13.6 ± 0.5
17:0	trace	trace	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.1	0.8 ± 0.0	0.5 ± 0.1	0.4 ± 0.0
18:0	5.1 ± 0.4	5.4 ± 0.8	4.8 ± 1.1	2.0 ± 0.2	7.5 ± 0.6	6.1 ± 0.3	6.3 ± 1.1	2.5 ± 0.6
20:0	trace	trace	trace	trace	trace	trace	trace	trace
Total monoenoic	54.1	53.1	66.5	54.0	45.2	42.1	59.7	59.8
16:1n-7	3.3 ± 0.3	3.9 ± 0.8	3.8 ± 0.6	7.3 ± 0.8	4.5 ± 0.2	5.3 ± 0.3	5.3 ± 0.7	5.2 ± 0.8
16:1n-5	trace	trace	trace	trace	trace	trace	trace	trace
18:1n-9	24.7 ± 3.9	26.6 ± 9.0	27.6 ± 3.7	14.6 ± 0.9	32.6 ± 1.5	26.3 ± 0.8	27.0 ± 2.9	19.0 ± 2.5
18:1n-7	4.3 ± 0.3	1.7 ± 1.2	2.7 ± 0.3	2.8 ± 0.2	2.7 ± 0.0	3.2 ± 0.2	2.8 ± 0.3	3.5 ± 0.3
18:1n-5	0.4 ± 0.0	0.4 ± 0.1	0.5 ± 0.1	0.8 ± 0.0	trace	0.4 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
20:1n-11	12.4 ± 2.5[b]	8.5 ± 3.5[b]	7.0 ± 2.1	10.4 ± 0.2[b]	3.7 ± 0.2[b]	1.3 ± 0.5	6.5 ± 2.0	8.3 ± 1.9
20:1n-9			5.7 ± 0.2			2.0 ± 0.2	3.2 ± 0.3	5.0 ± 1.0
20:1n-7	0.4 ± 0.1	trace	0.4 ± 0.1	0.4 ± 0.0	trace	trace	0.7 ± 0.0	0.4 ± 0.0
22:1n-11	7.2 ± 3.7	10.5 ± 5.8	14.8 ± 6.1	12.9 ± 2.1	0.9 ± 0.2	2.2 ± 0.5	7.4 ± 0.7	13.7 ± 0.9
22:1n-9	0.7 ± 0.4	0.7 ± 0.2	1.6 ± 0.6	3.1 ± 0.5	0.6 ± 0.1	0.4 ± 0.1	4.1 ± 1.2	2.4 ± 1.0
24:1n-9	0.7 ± 0.4	0.8 ± 0.3	2.3 ± 0.6	1.7 ± 0.1	0.3 ± 0.1	0.9 ± 0.3	2.1 ± 0.3	1.7 ± 0.3
Total polyenoic n-6 series	2.1	2.0	2.0	2.1	2.2	3.1	2.2	2.5
16:2n-6	0.6 ± 0.1	0.6 ± 0.0	0.7 ± 0.3	1.2 ± 0.0	0.7 ± 0.1	0.9 ± 0.3	1.2 ± 0.1	1.2 ± 0.2
18:2n-6	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.2	0.9 ± 0.1	0.8 ± 0.1	1.3 ± 0.2	0.7 ± 0.2	0.9 ± 0.1
20:4n-6	0.6 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	trace	0.7 ± 0.2	1.0 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
Total polyenoic n-3 series	16.8	17.2	9.3	16.9	15.3	17.6	7.4	12.7
18:3n-3	0.5 ± 0.1	0.6 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.5 ± 0.0	0.6 ± 0.1	0.3 ± 0.1	0.5 ± 0.1
18:4n-3	0.6 ± 0.2	0.8 ± 0.3	0.3 ± 0.1	1.3 ± 0.2	0.5 ± 0.1	0.9 ± 0.2	0.5 ± 0.1	0.9 ± 0.3
20:4n-3	1.3 ± 0.3	1.3 ± 0.1	0.5 ± 0.3	1.0 ± 0.1	0.8 ± 0.1	0.7 ± 0.2	0.5 ± 0.1	0.6 ± 0.7
20:5n-3	7.4 ± 1.4	6.0 ± 0.9	2.2 ± 0.5	8.2 ± 0.9	5.3 ± 0.4	6.2 ± 0.6	2.4 ± 0.7	4.7 ± 0.7
22:5n-3	1.7 ± 0.1	1.1 ± 0.3	0.8 ± 0.3	0.7 ± 0.1	1.2 ± 0.2	0.6 ± 0.2	0.4 ± 0.1	0.6 ± 0.1
22:6n-3	5.3 ± 0.9	7.5 ± 1.5	5.0 ± 1.5	5.3 ± 0.1	7.1 ± 1.1	8.6 ± 1.8	3.3 ± 0.6	5.4 ± 1.4

[a]Results are expressed as weight percentage of total fatty acids. Data are mean ± SD for three samples, "trace" denotes <0.3%. See Table 2 for species' complete names.
[b]Mixture of 20:1n-9 and 20:1n-11.

constituents among polyenoic fatty acids. All these major components were detected at a level of 3% or more of the total fatty acids in one or both samples, and the total amount of these major components was higher than 85% in each sample. Appreciable amounts of 14:0 (1.5–4.8%), 18:0 (2.0–7.5%), and 24:1 (0.3–2.3%) were also present. Interestingly, monoenoic acids such as 18:1 and 22:1 were also the major components in the total fatty acids of the Myctophids containing triacylglycerols as the major constituent similar to those in wax esters. The ratio of total monoene fatty acids reached to 42.1–66.5% of the total fatty acids (Table 4), and the major fatty acids of triacylglycerols in eight species contained considerable amounts of monoenes. Both the wax esters and the triacylglycerols in the Myctophid species contained only several percentage or less of each polyunsaturated fatty acids (PUFA) such as EPA and DHA, as shown in Tables 3 and 4, compared to the fatty acid compositions of other marine fish species, which are generally rich in PUFA (18). Therefore, there is little difference in the tendency of low total PUFA contents between the wax esters (three species) and the triacylglycerols (eight species). It is suggested that Myctophids that contain not only wax esters but also triacylglycerols as major component may have high proportions of monoenes in their lipids differing from other marine fish species.

High content of monoenes in lipids of Myctophids. It is concluded that the lipids in Myctophids caught in the oceanic front (mean seawater temperature 20°C) of the cold current of the Oyashio in northern Pacific Ocean contained high amounts of monoenes. Especially, 18:1, 20:1, and 22:1 were the dominant fats both for triacylglycerols and wax esters in the lipids of all samples, and polyunsaturated fats were found only in small amounts.

Similarity of the lipids between Myctophids and some surface pelagic fish species, and the effect of feeding habits on the high content of monoenes in the lipid of Myctophids. In general, fish lipids contain various amounts of many kinds of fatty acids (19); however, consistently high amounts of monoenoic fats are found in the lipids of some pelagic surface fish species such as saury (20), capelin (21,22), sprats (23), and herring (24, 25), whose lipids originate from their prey organisms such as zooplanktons (26–30). Ratnayake and Ackman (24,25) reported that these fishes actively prey on zooplankton, and pointed out that the planktonic fatty alco-

hols are assimilated efficiently and are converted directly to the fatty acids of the above pelagic fish species. Copepods and euphausiids are the most important prey on the basis of biomass, and some copepod species often contain large amounts of wax esters as energy reserves instead of triacylglycerols, and the monoene alcohols are generally the major constituents in the esters (26–30).

On the other hand, it is known that common Myctophids feed primarily on the zooplankton such as euphausiids and copepods, and these diets are diverse and overlap broadly. For example, Tyler and Pearcy (9) reported that the myctophids, such as genus *Diaphus* and *Stenobrachius,* mainly feed on copepod of the genus *Calanus* and euphausiid of the genus *Euphausia.*

In the present paper, the Myctophid species contained triacylglycerols and wax esters, and the fatty acid and alcohol compositions of these lipids were mainly composed of monoenes similar to those of the surface pelagic fish species. It is estimated that these deep-sea fishes accumulate in entirety the wax esters from their prey organisms, or transform the wax esters to the triacylglycerols without synthetic modification such as carbon chain elongation and desaturation of fats. The Myctophid species may prey on similar planktons as the pelagic fishes do because the fats of all the Myctophids examined consisted of 18:1, 20:1, and 22:1, being extremely similar to those of these pelagic fish species. It is suggested that the feeding habits of these eleven species of Myctophids caught in the transition zone between the Oyashio and the Kuroshio may be similar to those of the pelagic fish species such as saury caught in the same sea area, and it is assumed that the monoene fats in the wax esters of copepods, which may have a wide vertical distribution, are the precursors of the monoene fats of the Myctophids.

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Quantitative ^{31}P Nuclear Magnetic Resonance Analysis of the Phospholipids of Erythrocyte Membranes Using Detergent

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ABSTRACT: ^{31}P Nuclear magnetic resonance (NMR) spectra of human erythrocyte lysates dissolved in sodium cholate were acquired. The narrow resonances of phospholipids were mostly well resolved, allowing identification and accurate quantitative analysis of phospholipid classes of the erythrocyte membranes. The ether-linked phosphatidylethanolamine components of the erythrocyte membranes were identified, based on the removal of plasmalogens by acidolysis and of diacyl phospholipid species by degradation using phospholipase A_1. It was also shown that the introduction of double bonds on the acyl chains of phosphatidylcholine shifted the ^{31}P NMR resonances to lower frequencies. Quantitative analyses of phospholipids from the spectra were based on their apparent molar concentrations. The recoveries of phospholipids from erythrocytes were significantly higher than those using conventional extraction procedures.

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The importance of phospholipids in membrane structure and their significant role in many physiological processes are well known (1,2). Quantitative analysis of the phospholipid composition of human erythrocyte membranes is of particular interest in clinical investigations. It has been reported that in hyperlipidemic patients, the ethanolamine plasmalogen content of erythrocyte membranes is significantly decreased and the concentration of sphingomyelin (SPH) increased (3,4). Plasma high density lipoprotein levels in these patients are positively correlated with the content of ethanolamine plasmalogen in the erythrocyte membranes (3). A variation in outer-to-inner lamellar phospholipids of erythrocyte membranes in various clinical disorders, including coronary heart disease, also has been reported (5). These examples indicate why an accurate quantitative determination of individual phospholipid classes in erythrocyte membranes is important.

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Abbreviations: Lyso-PC, lysophosphatidylcholine; lyso-PE, lysophosphatidylethanolamine; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; $PC_{s,m}$, saturated and monounsaturated-diacyl phosphatidylcholine; PCp, polyunsaturated-diacyl phosphatidylcholine; PE, phosphatidylethanolamine; PLA_1, phospholipase A_1; PL, phospholipid; PS, phosphatidylserine; SPH, sphingomyelin.

A variety of methods have been employed for the quantitative analysis of phospholipids in erythrocyte membranes including thin-layer chromatography and high-performance liquid chromatography (6–10). These methods are usually multistep and require phospholipid extraction using organic solvent mixtures. ^{31}P and ^{1}H nuclear magnetic resonance (NMR) techniques also have been used for quantitative determination of erythrocyte phospholipids (11,12), but these methods have also involved sample extraction. Extraction of various phospholipids from erythrocyte membranes is time-consuming, and the recovery is usually influenced by the conditions of extraction. It has been shown that widely used solvents do not completely extract all phospholipid classes from erythrocyte membranes and the particular solvents must be selected to optimize the extent of extraction according to the phospholipid classes that are being analyzed (13).

In this study, we have quantified for the first time the concentrations of phospholipid classes in human erythrocyte membranes by using ^{31}P NMR and detergent. The values obtained were compared with those determined by techniques which involve lipid extraction. We have shown that the recoveries of phospholipid classes from erythrocytes were significantly higher by using detergent addition to erythrocytes than by using extraction methods. This novel method is a rapid, simple, one-step procedure, and is significantly more accurate than conventional analyses of erythrocyte phospholipids, which require complicated and time-consuming extractions.

In addition to this novel method of phospholipid quantification of erythrocyte membranes, we have further identified and determined the ether-linked phosphatidylethanolamine (PE) components of erythrocyte membranes based on the removal of plasmalogens by acidolysis and of diacyl phospholipid species by phospholipase A_1 (PLA_1) degradation. The values of PE components of erythrocytes obtained by using hydrolysis were also compared with those obtained by detergent addition to erythrocytes.

MATERIALS AND METHODS

Materials. Cholic acid (sodium salt), phosphatidylcholine (PC), PE, PE with 60% alkenylacyl PE (plasmalogen),

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lysophosphatidylcholine (lyso-PC), and phosphatidylserine (PS) were obtained from Sigma Chemical Co. (St. Louis, MO). D_2O (99.75%) was from the Australian Institute of Nuclear Science and Engineering (Lucas Heights, NSW, Australia). Deuterated methanol and chloroform were from Merck (Montreal, Canada). All other reagents were A.R. grade.

Sample preparation. Human erythrocytes were prepared from freshly drawn venous blood from a healthy donor and anticoagulated with heparin [~15 IU (mL whole blood)$^{-1}$]. Erythrocytes were then washed three times in isotonic saline (154 mM NaCl; 277 K, 5 vol). Cell suspensions were adjusted to hematocrits between 0.50 to 0.55 and were then subjected to tip sonication (Model B-12 Sonifier; Branson Sonic Power Co., Danbury, CT) for 10 s. For sodium cholate experiments, 2.5 mL of sonicated erythrocytes were transferred to 10-mm NMR tubes, and 0.5 mL Na cholate (with 5 mM EDTA) in D_2O was added with a final Na cholate concentration of 200 mM. The samples were bath sonicated for 2–5 min prior to NMR measurements. For lipid extractions, 0.5 mL of distilled water was added to 2.5 mL of sonicated erythrocyte sample, and the phospholipids were then extracted using chloroform/isopropanol and chloroform/methanol according to the methods of Rose and Oklander (14) and Bligh and Dyer (15), respectively, and dried under a stream of nitrogen. Dried lipids were dissolved in 3 mL of $CDCl_3/CD_3OD$ (2:1, vol/vol) for NMR measurements.

Hydrolysis of phospholipids. The methods of hydrolysis of plasmalogens and diacyl phospholipids by HCl and PLA_1, respectively, in human erythrocytes were based on those described by Diagne *et al.* (16). Fresh erythrocytes were washed in isotonic saline as described previously, and the lipids were extracted by the method of Rose and Oklander (14). Nonlipid contaminants were removed by partition against water (17), and the total lipid content was weighed. The samples were then divided into four parts and evaporated to dryness under vacuum in round-bottom flasks. One sample (control) was treated with Na cholate as described previously and was transferred to a 10-mm NMR tube. A second sample was inverted over a beaker of concentrated HCl for 10 min, flushed with nitrogen, and placed under vacuum for a further 10 min (HCl hydrolysis). The sample was treated with Na cholate before being transferred to an NMR tube. This procedure degrades the plasmalogen-containing phospholipids. Two other samples were sonicated in 0.2 M Tris-HCl buffer, pH 8.0, containing 2.4 mM sodium deoxycholate (approximately 400 nmol phospholipid per mL) for 30 s. The samples were then incubated for 3 h with a crude preparation of guinea pig pancreatic PLA_1 at 37°C in a shaking water bath. This treatment degrades the diacyl phospholipids, producing 2-acyl-lysophospholipids. The lipase was prepared according to the method of El Tamer *et al.* (18) and passed through a Sephadex G25 column (Pharmacia, Uppsala, Sweden). Sufficient PLA_1 enzyme activity was used in the incubation to completely hydrolyze an amount, similar to that in the extracts, of pure diacyl-PC standard to lyso-PC. After phospholipase treatment, the samples were either freeze-dried overnight or "Folch-washed" (17) and then dried under vacuum. One sample was then subjected to a further hydrolysis by exposure to HCl fumes, leaving only those phospholipids corresponding to the 1-alkyl-2-acyl species. Both samples were then supplemented with sodium cholate and were transferred to NMR tubes.

NMR spectroscopy. ^{31}P NMR spectra were recorded on a Bruker AMX-400 wide-bore spectrometer (Karlsruhe, Germany), operating at 161.98 MHz in the Fourier transform mode, with the probe temperature controlled to 25 ± 1°C. Broad-band proton irradiation was applied to eliminate ^1H–^{31}P NMR coupling. All measurements were done on 3-mL samples in 10-mm NMR tubes with D_2O ($CDCl_3$ in the case of extracts) as the internal field/frequency lock. Samples were spun at 10 Hz. Rapid pulsing using the method of Homer and Roberts (19) was used to acquire the spectra. Each spectrum was derived by Fourier transformation of 1600 summed transients with a spectral accumulation time of ~14 min. Other NMR parameters were as follows: transient-acquisition time, 0.5 s; data size, 16 k; 43° pulse, duration 11.5 μs; pulse delay, 1 ms. For fully relaxed ^{31}P NMR spectra of phospholipid mixtures in sodium cholate, a 24.1 μs (90°) pulse with a 7-s relaxation delay (~5 T_1) was used. No line-broadening was used for processing of the free induction decays of the spectra that are shown here. For T_1 measurements, the sample consisted of 2.4 mM phospholipids, 200 mM sodium cholate, and 1 mM EDTA in D_2O, and the measurements were made by using an inversion recovery pulse sequence with 160 transients per spectrum.

Quantitative analysis of phospholipids. The concentrations of phospholipids were calculated from the spectra by comparing the peak areas (intensities) of phospholipid resonances with those obtained for known concentrations of authentic compounds. In this manner, standard samples were prepared by adding a known mass of lyso-PC in D_2O to the samples that were treated with sodium cholate. The effective molecular weight of lyso-PC was determined by the comparison of the peak area of lyso-PC with that of a known amount of inorganic phosphate (dried at 110°C overnight) in a fully-relaxed ^{31}P NMR spectrum. The T_1 values of various phospholipid standards in sodium cholate at 25°C were measured; the values obtained were very similar, and varied in the narrow range between 1.2 and 1.3 s. However, lyso-PC had a longer T_1 value than the other phospholipids (1.42 s), presumably due to its smaller molecular size. Due to the similarity of the T_1 values and nuclear Overhauser effects (20) of the various phospholipids in mixed micelles, the peak areas in the fully relaxed spectrum remained very similar to those in the spectrum acquired using the rapid-pulsing procedure. However, the ratio between the areas of the lyso-PC in the fully relaxed and rapid-pulsing spectra was 1.1:1; thus, for quantitative analysis of phospholipids, the peak area of lyso-PC was corrected by multiplying by this factor. For quantification of phospholipid concentrations in extracts, PC was used as an internal standard, the effective molecular weight of PC was determined in a similar way to that of lyso-PC. Peak areas were determined using automatic Lorentzian deconvolution

rather than integration, due to the overlapping phosphorus resonances of some of the phospholipids.

RESULTS

^{31}P NMR analysis of erythrocyte phospholipids. A ^{31}P NMR spectrum obtained from a sample of sodium cholate-treated human hemolysate is shown in Figure 1. It is evident that several resonances can be assigned to the various phospholipid classes in human erythrocytes, whereas these resonances are totally absent in the relevant section of the ^{31}P NMR spectrum of untreated human erythrocytes (not shown). The resonances in Figure 1 included those due to inorganic phosphate at 2.76 ppm, SPH at 0.493 ppm, ether-linked PE (alkenylacyl-PE and alkylacyl-PE) at 0.457 ppm, diacyl-PE at 0.431, PS at 0.217 ppm, phosphatidylinositol at –0.01 ppm, $PC_{s,m}$ (saturated and monounsaturated-diacyl PC) at –0.115 ppm and PC_p (polyunsaturated-diacyl PC) at –0.135 ppm. These assignments were confirmed: (i) by adding authentic phospholipids and phosphate to the sodium cholate-treated lysate sample and observing the increase in signal amplitude and intensity; and (ii) by selective hydrolysis of alkenylacyl (plasmalogen) and diacyl phospholipids with HCl and PLA_1, respectively. However, $PC_{s,m}$ and PC_p resonances were only tentatively assigned (21) (mol% of total phospholipids for $PC_{s,m}$ and PC_p were 18.8 ± 1.5 and 11.5 ± 0.9, respectively). Chemical shift assignments were "externally" referenced relative to 85% orthophosphoric acid at 0.000 ppm.

^{31}P NMR spectra of total lipid extracts from human erythrocytes (with an identical hematocrit to that of sodium cholatetreated erythrocytes) in $CDCl_3/CD_3OD$ (2:1, vol/vol) were also obtained (the spectra are shown in Fig. 1) in which only four classes of phospholipid resonances, including PC, PS, SPH, and PE (total), were resolved.

The molar concentrations of individual phospholipid classes of human erythrocytes determined from ^{31}P NMR

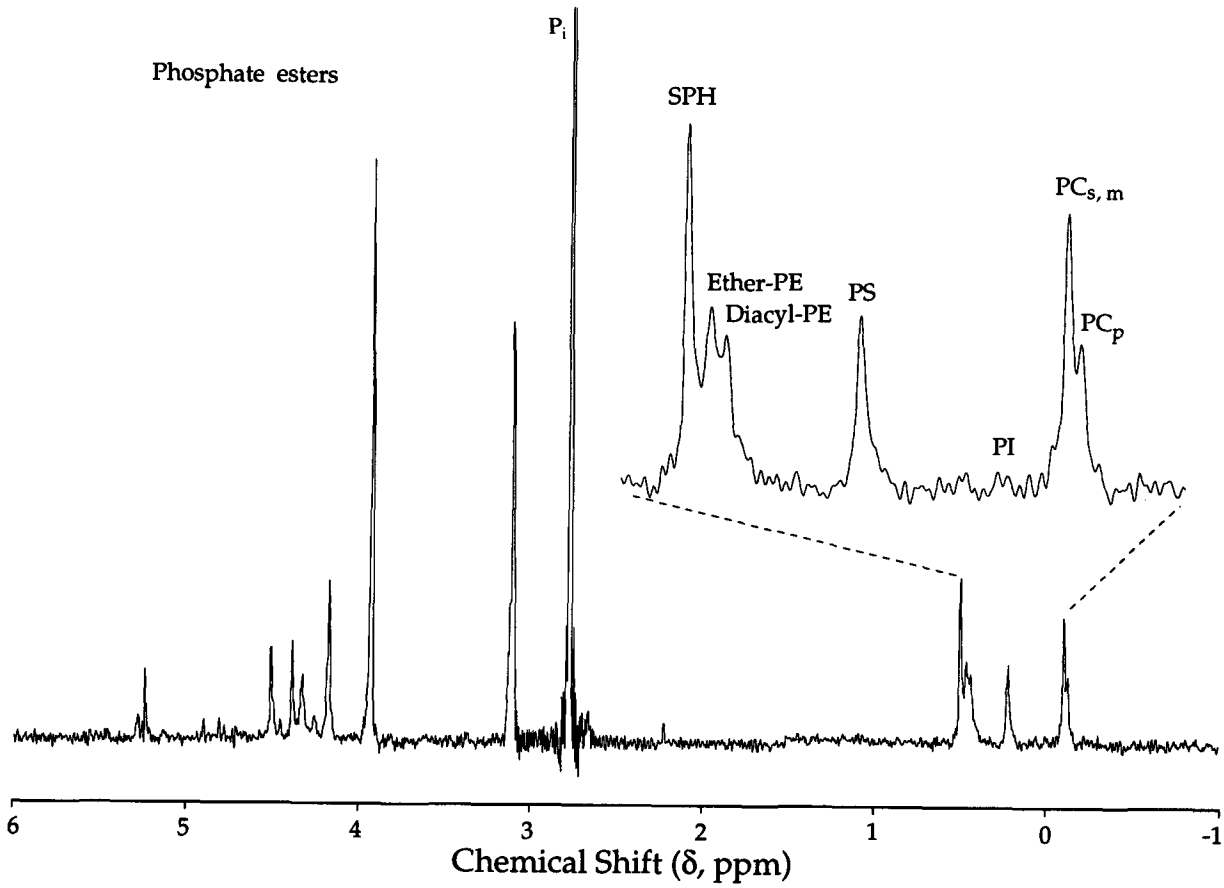


FIG. 1. ^{31}P Nuclear magnetic resonance (NMR) spectrum of sodium cholate-treated human hemolysate. Resonance assignments for the phospholipids indicated on the spectra were confirmed by adding authentic phospholipids to the NMR samples; and selective hydrolysis of alkenylacyl (plasmalogen) and diacyl phospholipids with HCl and phospholipase A_1, respectively. $PC_{s,m}$ and PC_p resonances were tentatively assigned. NMR parameters are given in the Materials and Methods section; pH of samples was ~8. PC_p, polyunsaturated-diacyl phosphatidylcholine; $PC_{s,m}$, saturated and monounsaturated-diacyl phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; SPH, sphingomyelin; and P_i, inorganic phosphate.

TABLE 1
Comparison of Concentration of Phospholipid Classes from Human Erythrocytes[a] Using Na Cholate Addition and Extraction Methods by ^{31}P Nuclear Magnetic Resonance

	Method		
Phospholipid[b]	Na cholate addition[c]	Rose extraction[d]	Bligh extraction[e]
SPH	689 ± 20 (100%)[f]	640 ± 18 (93%)	545 ± 15 (79%)
Total PE	1040 ± 80 (100%)	804 ± 64 (80%)	670 ± 50 (66%)
PS	446 ± 27 (100%)	326 ± 20 (673%)	165 ± 10 (37%)
Total PC	944 ± 9 (100%)	948 ± 10 (100%)	737 ± 8 (78%)
Total PL	3120 ± 60 (100%)	2720 ± 50 (87%)	2120 ± 40 (68%)

[a]Values are expressed in μmol (L packed erythrocytes)$^{-1}$ ± SD.
[b]SPH, sphingomyelin; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; PL, phospholipid.
[c]For details of the experimental procedure see the Materials and Methods section.
[d]Rose and Oklander (Ref. 14). Human erythrocytes were extracted using chloroform/isopropanol (7:11, vol/vol).
[e]Bligh and Dyer (Ref. 15). Human erythrocytes were extracted using chloroform/methanol (2:1, vol/vol).
[f]Relative recovery of phospholipid classes. Recoveries by the sodium cholate addition method were 100% and thus serve as the "standard."

spectra of extracts and sodium cholate-treated erythrocytes are given in Table 1. The recoveries of phospholipids from erythrocytes, assessed by using known amounts of added exogenous phospholipids and sodium cholate additions, were compared with those obtained from extracted erythrocytes. As is evident, the recoveries of the phospholipids were significantly greater with the detergent method.

Identification of ether-linked phospholipids. Figure 2A shows a ^{31}P NMR spectrum of an extract of erythrocytes treated with sodium cholate. The spectrum is virtually identical to that in Figure 1, with a slight improvement in the resolution of ether-linked PE resonances (resonances 2 and 3 belong to alkenylacyl-PE and alkylacyl-PE, respectively). When an acidic treatment by HCl fumes was carried out, only alkenylacyl-PE (resonance 2) disappeared from the spectrum (Fig. 2B); two additional resonances appeared and corresponded to lysophosphatidylethanolamine (lyso-PE) isomers (resonances 2a and 2b) due to the specific splitting of the 1-alkenyl bond. The assignment of the resonances was confirmed by HCl treatment of a mixture of 30% PE and 60% alkenylacyl-PE standard and observing the appearance of lyso-PE resonances. The areas of the alkenylacyl-PE resonance in Figure 2A, and those assigned to lyso-PE in Figure 2B, were also identical. The areas of other phospholipid resonances remained the same, both A and B of Figures. Upon PLA_1 treatments, all resonances assigned to PC, PS, and diacyl-PE disappeared (Fig. 1). The resonances corresponding to the lysoderivatives of most phospholipids were absent in the ^{31}P NMR spectra; this is because the water-miscible lysophospholipids were eliminated during the separation of the aqueous phase by using the Folch method. However, a small amount of lyso-PC was recovered, with the resonance appearing in the PE region of the spectrum, which was superimposed on the alkenylacyl-PE resonance. In order to identify those phospholipids that correspond to the 1-alkyl-2-acyl

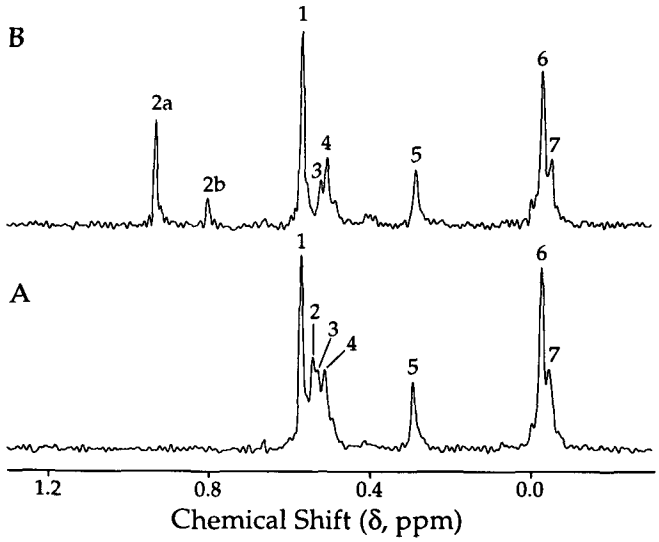


FIG. 2. ^{31}P NMR spectra of erythrocytes extract: A, treated with sodium cholate; B, treated by HCl fumes and then solubilized in sodium cholate. Assignments: 1, SPH; 2, ether-PE; 2a and 2b, lyso-PE isomers; 3, alkylacyl-PE; 4, diacyl-PE; 5, PS; 6, $PC_{s,m}$, 7, PC_p. Abbreviations as in Figure 1. Further experimental details are given in the Materials and Methods section.

species, experiments were also conducted where the samples were subjected to HCl hydrolysis after PLA_1 treatments (samples were freeze-dried overnight after treatment with PLA_1; see the Materials and Methods section). Apart from a resonance that appeared as a shoulder to the lyso-PC resonance in the ^{31}P NMR spectrum which was assigned to the alkylacyl-PE (Fig. 2), no other resonances in Figure 1 were detectable that could correspond to the 1-alkyl-2-acyl species of other phospholipids. Table 2 gives the content of PE of erythrocytes determined directly from ^{31}P NMR spectra of sodium cholate- treated erythrocytes (such as Fig. 1) and those ob-

TABLE 2
Phosphatidylethanolamine Composition (%)[a] of Human Erythrocytes, Obtained by ^{31}P Nuclear Magnetic Resonance (NMR) Analysis

Phosphatidylethanolamine	Determined by hydrolysis[b]	Determined directly[c]
Diacyl	13.2 ± 1.6	12.2 ± 1
Alkenylacyl (plasmalogen)	15.9 ± 0.6	—
Alkylacyl	2.5 ± 0.4	—
Ether-linked (alkenylacyl + alkylacyl)	18.4 ± 0.5	21.2 ± 1.7
Total	32 ± 1.8	33 ± 2.7

[a]Values are expressed as mol% ± SD of total phospholipids and are the average of three determinations on the same blood sample.
[b]Values were determined by hydrolysis of erythrocytes extract using HCl and PLA_1. For details of the experimental procedure, see the Materials and Methods section.
[c]Values were determined directly by dissolving erythrocyte lysates in detergent and acquiring ^{31}P NMR spectra.

tained from ^{31}P NMR spectra of erythrocyte extracts hydrolyzed with HCl (such as Fig. 2B). As can be seen, the values obtained for diacyl and ether-linked PE were similar.

DISCUSSION

Detergent was used in conjunction with ^{31}P NMR for the quantitative analysis of the phospholipid composition of human erythrocytes. The "detection levels" of phospholipids obtained with extraction of erythrocytes were significantly lower than those obtained by using the method of sodium cholate addition (Table 1). It has been shown by Eder *et al.* (13) that the recoveries of phospholipids from erythrocytes by using various extraction methods are influenced by the nature of the solvents, the duration of extraction, the temperature of extraction, and mechanical treatment of the sample. They also observed that recoveries of PC and SPH are not influenced by the conditions of extraction as much as those of PS and PE. It seems that this is related to the specific location of the phospholipids in the membrane; PC and SPH are located primarily in the outer leaflet of the bilayer, whereas PE and PS are located in the inner leaflet (13,22). A higher portion of saturated fatty acids in PC and SPH may be an additional factor influencing the higher extent of recovery of these phospholipids (13). Indeed, our present results show (Table 1) that by using extraction (chloroform/isopropanol, 7:11), only PC and SPH have high recoveries and these are comparable with those obtained by sodium cholate addition to the erythrocytes. The recoveries of PS and PE, on the other hand, were very poor by extraction (Table 1), especially using the Bligh and Dyer method (15) (chloroform/methanol, 2:1, vol/vol). Apart from differences in extraction conditions, the Bligh and Dyer method (15) requires filtration and phase separation, whereas in the Rose and Oklander method (14) (if NMR is used for analysis), the extract can be sampled directly without separation. For extraction of phospholipids from erythrocytes, Eder *et al.* (13) used ten binary and single-solvent systems with different extraction conditions. They concluded that, although the widely used methods gave incomplete extraction of all phospholipid classes from erythrocytes, hexane/isopropanol (3:2, vol/vol) is the most suitable solvent mixture for extraction of all phospholipids; and boiling of the sample during its processing is required for complete extraction of PE and PS. In the present study, for the comparison of recoveries of erythrocyte phospholipids (between detergent addition and extraction methods), we selected the most widely used extraction procedures (see the Materials and Methods section). Thus, we conclude that the extraction methods applied to erythrocytes may not reliably yield quantitative results of most erythrocyte phospholipid classes.

Although detergents such as Triton X-100 and sodium cholate have already been used for phospholipid analysis of human blood plasma (17,23,24) and amniotic fluid (21), the use of detergents for quantitative analysis of cell membrane phospholipids has not been reported by other workers (see our recent application to the analysis of animal red cell membranes; Ref. 25). The chemical shifts of phospholipids in Figure 1 are in good agreement with those reported by others for phospholipid micelles in detergent (21–23). The chemical shifts are also pH dependent, due to the state of ionization of the phosphate groups (24); however, between pH ~3 and ~9, they are unchanged (24). The narrow ^{31}P NMR line widths of erythrocyte phospholipids in sodium cholate (Fig. 1), which result from efficient averaging of chemical shift anisotropy and of dipolar interactions in small micelles (24), allow the individual phospholipid resonances to be at least moderately well resolved.

Although by acidic hydrolysis of alkenylacyl-PE, 2-acyl-lyso PE was expected to be the product, and we observed the appearance of two resonances (Fig. 2B) that were separated by 0.13 ppm. These two resonances also appeared with HCl treatment of standard alkenylacyl-PE. The rearrangement of lysophospholipids *via* acyl and phosphoryl migration has been reported (26). Plückthun and Dennis (26) by using ^{31}P NMR have shown that, at alkaline pH values, lysophospholipids can exist in the form of 1-acyl and 2-acyl isomers. At acidic pH values, however, several compounds were formed from 2-acyl PC, due to the hydrolysis of the choline group, acyl hydrolysis and migration, and phosphoryl migration. They were unable to identify all the migration products at acidic pH, partly because of some signal overlap and partly due to the complexity of the reactions. Our aim in this study was only to identify the plasmalogen content of PE by acidic hydrolysis; thus, we assigned the resonances in Figure 2B as lyso-PE isomers.

$PC_{s,m}$ and PC_p resonances in Figure 1 were tentatively assigned to saturated + monounsaturated and polyunsaturated acyl-PC, respectively. Pearce and Komoroski (21) have shown that the presence of double bonds in the acyl chains of phospholipids (dissolved in bile salts) induces small, but consistent, low frequency (upfield) shifts relative to the corresponding disaturated acyl phospholipid ^{31}P NMR signals. They proposed that the composite PC signal can be segre-

gated into three regions, starting at lower field-disaturated acyl PC, saturated–unsaturated acyl PC, and diunsaturated PC, with the resonances separated by 0.02 ppm from each other. PC_p resonates at 0.02 ppm to low frequency (upfield) from $PC_{s,m}$ (Fig. 1), with the percentages of PC_p and $PC_{s,m}$ to total PC being 62 and 38%, respectively. These values also were similar to those that had been reported by others (27,28). The fatty acid contents of $PC_{s,m}$ and PC_p in human erythrocytes are predominantly 16:0, 18:0, 18:1 and 18:2, 20:4, respectively (27,28). Except for a small amount of linoleic acid in position 1, all the polyunsaturated fatty acids are located in position 2 (28).

The resonances for diacyl PE (unlike those for diacyl PC) were unresolved (Fig. 1). This was probably because the acyl chains of PE (and of PS) in human erythrocyte membranes are highly unsaturated compared with those of PC (28). On the other hand, the effect of acyl unsaturation on the chemical shift of the diacyl PE resonance is smaller than that on the chemical shift of the diacyl PC resonance in detergent (21). However, the small overlapping resonances, to low frequency from diacyl PE and PS (Fig. 1), may belong to their polyunsaturated fatty acid species as these phospholipids contain high amounts of 20:4 fatty acids (28).

In conclusion, we present here a convenient ^{31}P NMR method for quantitative analysis of phospholipid classes in human erythrocytes using sodium cholate to bring about spectral resolution. The significant advantages of this method are its speed, accuracy, and simplicity. The method needs minimal sample handling and the whole spectral analysis can be done in a very short time (~14 min). These features make the method ideal for studying the individual phospholipid changes in erythrocytes from humans and other animals, and presumably also in other cells, and should be valuable in clinical investigations for diagnosis of various diseases, especially cardiovascular ones.

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n-3 Polyunsaturated Fatty Acid-Induced Changes in the Molecular Species Composition of Diradylglycerol in Murine Peritoneal Macrophages Remain Stable During Incubation *ex vivo*

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ABSTRACT: Three- to four-week-old C57BL/6 mice were maintained for four weeks on diets in which the 10% lipid component was the ethyl esters of either corn oil or n-3 polyunsaturated fatty acids (n-3 PUFA). Incubation of macrophages *ex vivo* for 14 h, a normal incubation time used for macrophage studies, in the absence of fetal calf serum, did not diminish the extent of incorporation of 20:5n-3 (eicosapentaenoic acid) and 22:6n-3 (docosahexaenoic acid) moieties into membrane phospholipids and into diradylglycerol (DG) relative to that after a very abbreviated incubation time. We conclude that studies examining the effects of dietary n-3 PUFA on DG formation and related physiological effects in macrophages can be performed after a normal *ex vivo* incubation of at least 14 h without experiencing a significant loss of incorporated n-3 PUFA.
Lipids 31, 771–776 (1996).

Humans are incapable of *de novo* synthesis of n-3 polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Incorporation of these PUFA into cell membrane lipids reflects their dietary intake (1–3). However, functional studies on lipid second messengers conducted *ex vivo* after manipulation of dietary PUFA content are potentially problematic because a rapid loss of the diet-induced changes in fatty acid composition might occur during the customary prolonged (12–16 h) incubation of adhered macrophage monolayers *ex vivo* (4–8). A number of physiological functions of macrophages require 8–16 h incubation *ex vivo* to reach stable basal levels (4), but the stability of phospholipid (PL) and diradylglycerol (DG) molecular species compositions over this prolonged interval is unknown. In the present study, we investigated to what extent previously described dietary n-3 PUFA-induced changes in PL and DG molecular species in murine peritoneal macrophages (3) remain stable after a customary prolonged incubation period *ex vivo* relative to a much more abbreviated incubation. Our results define a basis of validity on which functional studies of the effects of dietary n-3 PUFA may be performed *ex vivo*.

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Abbreviations: AA, arachidonic acid; COEE, corn oil ethyl ester concentrate; DG, diradylglycerol; dfcs, heat-deactivated fetal calf serum; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PL, phospholipids; PMA, phorbol myristate acetate; PUFA, polyunsaturated fatty acids; n-3EE, n-3 fatty acid ethyl ester concentrate†.

MATERIALS AND METHODS

Animal care and diets. Ethyl ester concentrates were graciously supplied by the NIH/NOAA Fish Oil Test Materials Program (Charleston, SC). Pathogen-free 3–4-week-old C57BL/6 mice (4–5/cage) (Charles River Laboratory, Montreal, Quebec, Canada) were assigned to either corn oil ethyl ester concentrate (COEE) or n-3 fatty acid ethyl ester (n-3EE) diet groups for four weeks, as previously described (3), and allowed access to food and water *ad libitum*. Mice were individually weighed on day 0 and weekly thereafter to monitor growth.

Macrophage culture and stimulation. Mice were injected intraperitoneally with 1 mL brewer's thioglycollate (DIFCO, Detroit, MI). Macrophages were harvested and washed as described previously (3). For quantitative analyses of total DG mass, macrophage monolayers (2×10^6/9.62 cm^2 well) were adhered for 2 h, washed and cultured for an additional 2 h (abbreviated incubation) or 14 h (standard incubation). For DG and PL molecular species analyses, macrophage monolayers (15×10^6/16 cm^2 well) were similarly adhered and incubated. The culture medium was RPMI 1640 with (+dfcs) or without (–dfcs) 10% heat-deactivated fetal calf serum (dfcs), as indicated. Macrophage monolayers were then washed with Hank's Balanced Salt Solution containing 10 mmol/L HEPES, pH 7.4, and were stimulated for 10 min with either (PAF) (0.1 μM), ionomycin (10 μM), phorbol myristate acetate (PMA) (0.1 μg/mL), or HEPES-buffered Hank's Balanced Salt Solution control, as described previously (3). All conditions were repeated in duplicate or triplicate wells for each experiment.

Lipid analyses. Lipids were extracted by the Bligh and Dyer method (9). For total DG mass, DG was derivatized to

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phosphatidic acid, purified by thin-layer chromatography, and quantitated as described previously (3). For DG molecular species composition, DG was derivatized to dimethylphosphatidic acid and separated into molecular species fractions by argentation thin-layer chromatography as described (3). PL classes were separated and hydrolyzed to DG, then further analyzed as DG for molecular species fractions.

Statistical analysis. Results are expressed as mean ± SEM or as mean and range, as indicated. Statistical significance of differences between experimental conditions was tested by Student's two-tailed *t*-test using the Fig.P computer program (version 6.0c; Biosoft, Cambridge, United Kingdom).

RESULTS

Animal growth. Mice gained approximately 12 g after four weeks in both the n-3EE and COEE diet groups. Between diet groups, weights were not statistically significant at baseline or at weekly weighings.

The effects of incubation time, with and without fetal calf serum, on the total mass of DG. Mice were fed either n-3EE or COEE diets for four weeks, after which peritoneal macrophages were elicited, harvested, adhered for 2 h, and incubated *ex vivo* for an abbreviated (2-h) or a standard (14-h) duration, in either +dfcs or -dfcs medium. Macrophages were then left unstimulated to determine total basal DG mass or stimulated with PAF (0.1 μM) for 10 min. In the n-3EE diet group (Fig. 1A), basal DG mass was lower after the abbreviated than after the standard incubation time in both –dfcs medium (27.7 ± 1.7 pmol/10^6 macrophages compared to 31.9 ± 2.9, n.s.) and in +dfcs medium (30.5 ± 0.1 pmol/10^6 macrophages compared to 39.8 ± 0.9, $P < 0.05$). Similarly, after macrophage stimulation with PAF (Fig. 1A), DG mass was lower after abbreviated than after standard incubation time in both –dfcs medium (43.7 ± 5.4 pmol/10^6 macrophages compared to 45.6 ± 10.8, n.s.) and in +dfcs medium (66.5 ± 1.1 pmol/10^6 macrophages compared to 77.3 ± 8.1, $P < 0.05$). However, in the COEE diet group (Fig. 1B), both basal and PAF-stimulated DG mass were similar after abbreviated and standard incubation times in both -dfcs and +dfcs media.

The effects of incubation time, with and without fetal calf serum, on the molecular species composition of DG. Macrophages were prepared, incubated, and stimulated as described. Under both basal and PAF-stimulated conditions, our results (Fig. 2) generally show that the proportions of molecular species of DG after the standard incubation time did not differ significantly from the proportions present after only the abbreviated incubation time. Exceptions to this were seen only in +dfcs medium as follows: (i) In the n-3EE diet group (Fig. 2A), the fraction of basal DG with five double bonds (containing EPA) was 36.1–36.5 mol% after abbreviated incubation in +dfcs medium, but was lower by about 17% (30.7–30.9 mol%) after standard incubation (Fig. 2A,b). This difference was associated with a reciprocal change in DG species with four double bonds [containing arachidonic acid (AA)], whose proportion was 20.7–21.0 mol% after abbreviated incubation, but was 19% higher (26.6–28.7 mol%) after standard incubation (Fig. 2A,a). (ii) In the COEE diet group (Fig. 2B), the fraction of basal DG with four double bonds (containing AA) was 40.7–43.1 mol% after abbreviated incubation in +dfcs medium, but was 25% higher (54.2–57.4 mol%) after standard incubation (Fig. 2B,a). This difference was associated with a reciprocal change in the fraction of DG with six double bonds (containing DHA), which was 5.3–6.2

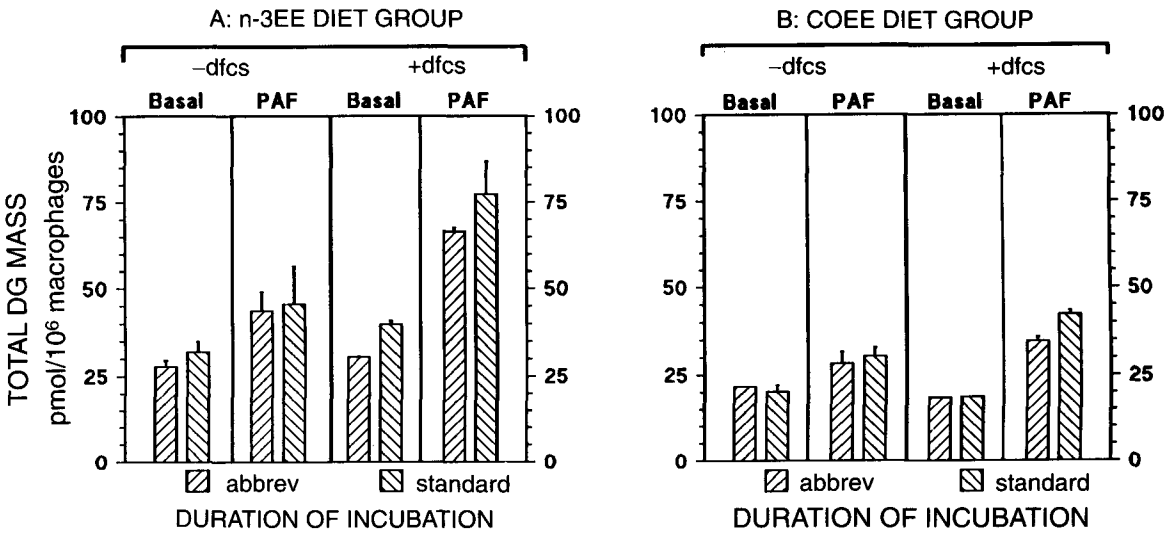


FIG. 1. The effects of incubation time in the presence and absence of heat-deactivated fetal calf serum (dfcs) on the total mass of diradylglycerol (DG) in murine peritoneal macrophages. Mice were fed diets containing either A: n-3 fatty acid ethyl ester concentrate (n-3EE) or B: corn oil ethyl ester concentrate (COEE) for four weeks. Macrophages were harvested, adhered for 2 h, incubated for an additional 2 (abbrev) or 14 (standard) h in either the absence (–dfcs) or the presence (+dfcs) of fetal calf serum in the culture media, and assayed for DG mass either under basal conditions or after a 10-min 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) stimulation, as described in the Materials and Methods section. Results are expressed as pmol DG/10^6 macrophages, mean ± SEM ($n = 4$).

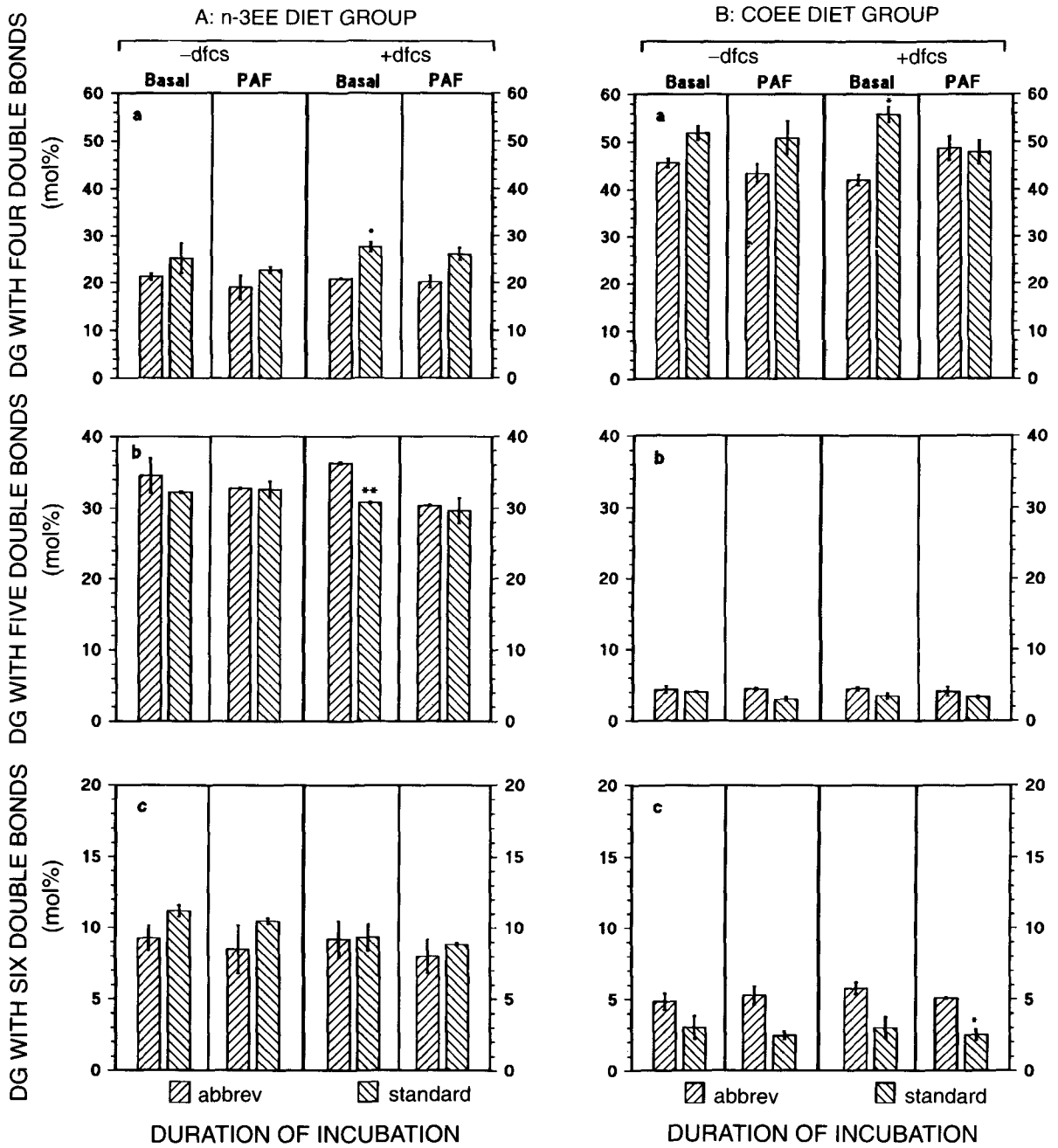


FIG. 2. The effects of incubation time in the presence and absence of dfcs on the molecular species composition of DG in murine peritoneal macrophages. Mice were fed diets containing either A: n-3EE or B: COEE for four weeks. Macrophages were prepared as in Figure 1 and assayed for DG molecular species composition, as described in the Materials and Methods section. Results for basal and for PAF-stimulated DG after 2 (abbrev) or 14 (standard) h of incubation without (–dfcs) or with (+dfcs) fetal calf serum are shown for molecular species fractions with (a) four, (b) five, and (c) six double bonds. Results are expressed as mol% of total DG, mean and range (n = 2). See Figure 1 for other abbreviations.

mol% after abbreviated incubation, but was lower by approximately 50% (2.3–3.8 mol%) after the standard incubation time (Fig. 2B,c). In summary, significant differences in the molecular species composition of DG were not found between abbreviated and standard incubation times unless dfcs was present in the culture media.

These results confirmed our concerns that supplementation of culture media with dfcs may alter the molecular species composition of DG that results from a diet with a controlled lipid content. The remaining experiments were therefore conducted using –dfcs medium. Our results (Fig. 3) show that within each diet group and for each macrophage stimulus, the distinctive proportions of DG molecular species with four, five, and six double bonds measured after standard incubation were not significantly different from those measured after abbreviated incubation. An isolated exception is the results with PMA stimulation in the COEE diet group (Fig. 3B,a), where the mol% of species with four double bonds

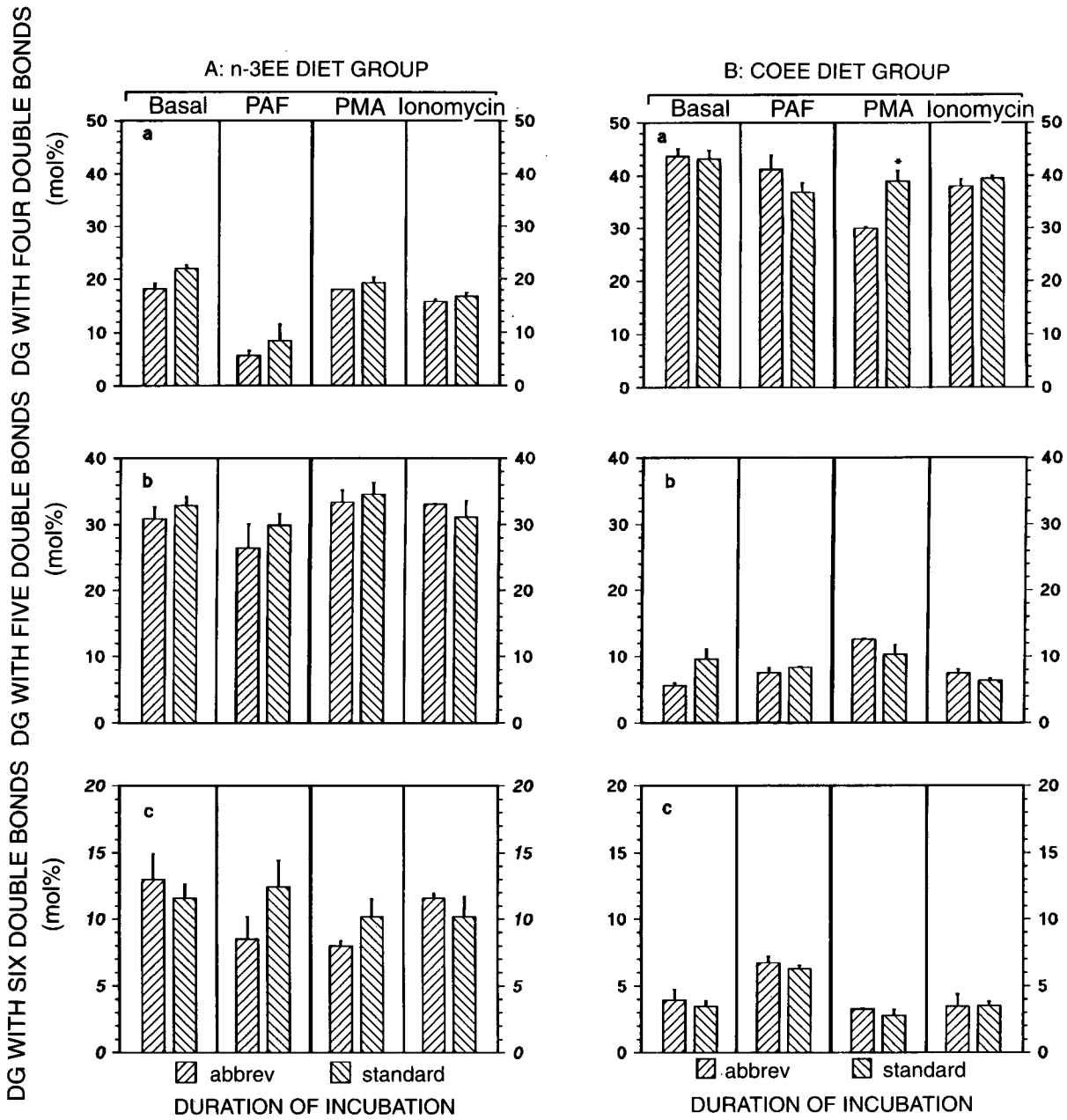


FIG. 3. The effects of incubation time on the molecular species composition of DG in variously stimulated murine peritoneal macrophages. Mice were fed diets containing A: n-3EE or B: COEE for four weeks. Macrophages were harvested, adhered for 2 h, incubated in media without dfcs for an additional 2 (abbrev) or 14 (standard) h and stimulated as described in the Materials and Methods section. Molecular species compositions of DG were analyzed as described in the Materials and Methods section. Results are shown for basal (n = 18) and agonist-stimulated conditions [PAF, 0.1 μM; phorbol myristate acetate (PMA), 0.1 μg/mL; and ionomycin, 10 μM; n = 6 each] for species with (a) four, (b) five, and (c) six double bonds. Results are expressed as mol% of total DG, mean ± SEM. *$P < 0.02$ relative to 2-h incubation. See Figure 1 for other abbreviations.

after abbreviated incubation (30 mol%) was lower than after standard incubation (40 mol%), the difference being due to a greater fraction of species with lesser degrees of unsaturation.

The effects of incubation time on the molecular species composition of PL classes. As DG is formed from membrane PL, and the results show a stability of DG molecular species composition over a period of standard incubation *ex vivo*, we wished to determine whether a corresponding stability of the dietary n-3 PUFA-induced changes in membrane PL also could be demonstrated.

Murine peritoneal macrophages were prepared after four weeks of either n-3EE or COEE diets, as described in the Materials and Methods section. After 2-h adherence and additional incubation *ex vivo* without dfcs for either an abbreviated (2-h) or a standard (14-h) duration, membrane PL classes phosphatidylcholine (n = 12), phosphatidylinositol (n = 8),

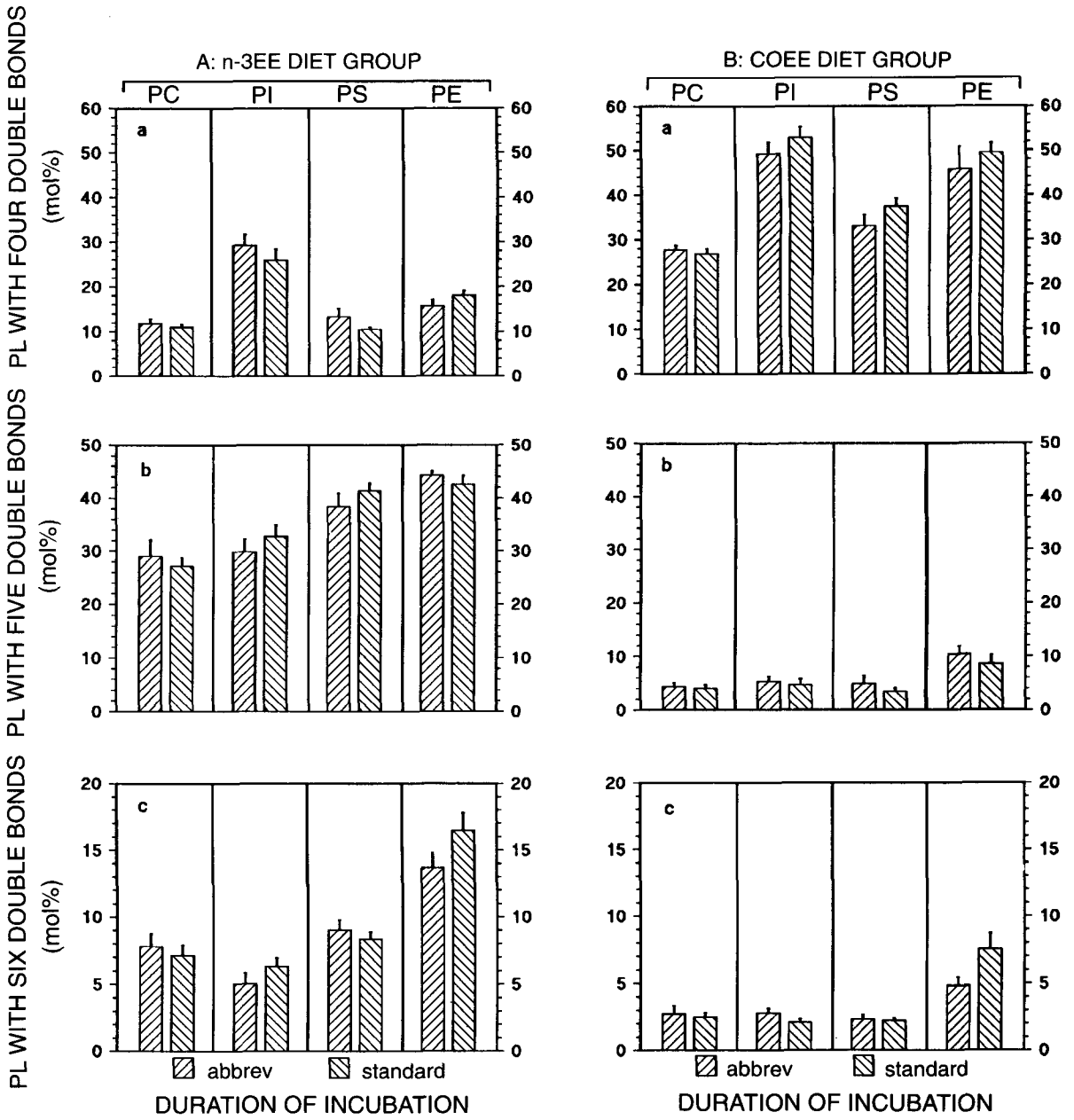


FIG. 4. The effects of incubation time on the molecular species composition of phospholipid (PL) classes in murine peritoneal macrophages. Mice were fed diets containing A: n-3EE or B: COEE for four weeks. Macrophages were prepared as in Figure 3 and incubated in media without dfcs for 2 (abbrev) or 14 (standard) h. PL classes were separated, and their molecular species compositions were analyzed as described in the Materials and Methods section. Results are shown for phosphatidylcholine (PC; $n = 12$), phosphatidylinositol (PI; $n = 8$), phosphatidylserine (PS; $n = 8$), and phosphatidylethanolamine (PE; $n = 10$) for species with (a) four, (b) five, and (c) six double bonds. Results are expressed as mol% of each PL, mean ± SEM. See Figure 1 for other abbreviations.

phosphatidylserine ($n = 8$), and phosphatidylethanolamine ($n = 10$) were extracted, separated, and analyzed for molecular species fractions (Fig. 4). Characteristic differences between the PL classes in their molecular species profiles are as previously described (3). For each PL class, however, the characteristic species distribution found after abbreviated incubation remained entirely preserved after standard incubation. No discernible trend over time was observed within any PL class in any molecular species fraction, in either the n-3EE (Fig. 3A) or the COEE (Fig. 3B) diet group. These results show that dietary n-3 PUFA-induced enrichment of macrophage membrane PL with EPA and DHA does not significantly degrade over the standard *ex vivo* incubation time.

DISCUSSION

It is a significant concern that cell membrane lipid modifications achieved by dietary changes *in vivo* might quickly be lost during incubation of cells in *ex vivo* experiments. In the current study, we characterized the effects of incubation time *ex vivo* on molecular species distributions of DG and PL.

We found that the total DG mass in macrophages in the n-3EE diet group tended to be greater after the standard than after the abbreviated incubation when dfcs was present in the culture media. It was stable under most other conditions (Fig. 1A). However, many other normal physiological functions of macrophages equilibrate only after several hours of incubation *ex vivo* (4). Our results (Fig. 1) suggest that total DG mass is similarly not yet stable after abbreviated incubation (2 h), particularly if dfcs is present in the culture media. For this reason, it is often important that macrophage monolayers not be studied *ex vivo* immediately after achieving adherence.

Molecular species of DG with EPA at the *sn*-2 position were diminished after standard incubation *ex vivo* in +dfcs medium, relative to abbreviated incubation, but this diminution was abolished in -dfcs medium (Fig. 2A,b). Furthermore, under both basal conditions and after stimulation of macrophages with either the physiological agonist, PAF, the tumor-promoting phorbol ester, PMA, or the divalent cation ionophore, ionomycin, we found that the proportions of DG molecular species studied in –dfcs medium were not different if the duration of *ex vivo* incubation was abbreviated to 2 h or was a standard 14 h (Fig. 3). Molecular species profiles of the PL classes phosphatidylcholine, phosphatidylinositol, phosphatidylserine, and phosphatidylethanolamine were similarly stable over the duration of the standard incubation (Fig. 4). In other words, there was no evidence of degradation of the lipid modifications that had previously been achieved *in vivo*. This PL stability presumably underlies the stability of the DG molecular species profiles discussed here.

We conclude that, at least under the conditions reported in this study, lipid modifications of macrophage membranes that are achieved *in vivo* after four weeks of dietary enrichment with n-3 PUFA are not lost *ex vivo* to any significant degree over the standard 14-h incubation time. These observations validate the use of *ex vivo* studies of macrophages to assess functional implications of dietary n-3 PUFA enrichment at the cellular level.

ACKNOWLEDGMENTS

This work was supported by grants from the Arthritis Society of Canada. RJS is the recipient of a Career Award from the Pharmaceutical Manufacturers' Association of Canada-Health Research Foundation and the Medical Research Council of Canada. PAM is the recipient of a Graduate Student Fellowship from the Pharmaceutical Manufacturers' Association of Canada-Health Research Foundation and the Medical Research Council of Canada.

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ANNOUNCEMENTS

SPECIAL *LIPIDS* SUPPLEMENT—PROCEEDINGS OF THE ISSFAL 2ND INTERNATIONAL CONGRESS

Extra copies of the March *Lipids* Supplement—"Fatty Acids and Lipids from Cell Biology to Human Disease," the Proceedings of the 2nd International Congress of the ISSFAL (International Society for the Study of Fatty Acids and Lipids)—which includes 57 full manuscripts from participants in oral presentations at the Congress (332 total pages), are available for individual purchase at $60 each. Information: AOCS Press, P.O. Box 3489, Champaign, IL 61826-3489 (Phone: 1-217-359-2344, extension 128; Fax: 1-217-351-8091).

CALENDAR

September 1996

37th International Conference on the Biochemistry of Lipids, AntwerpUniversity Center, University of Antwerp, Sept. 4–7, 1996. Symposia: enzymes and transfer proteins related to lipoprotein metabolism, aspects of protein lipidation, and peroxisomal lipid metabolism. Oral and poster communications covering the themes of the symposia and miscellaneous topics of the broad field of lipid biochemistry. Information: Professor Doctor A. Lagrou, RUCA-Lab Human Biochemistry, Department BFG, University of Antwerp, Groenenborgerlaan 171, B2020 Antwerp, Belgium (Phone: 3 2180329; Fax: 3 2180326).

7th International Congress on Phospholipids, Sept. 9–11, 1996, Brussels, Belgium. Program topic areas include Growth, Death and Cancer; Choline, Choline Phospholipids and the Brain; Importance of Phospholipids in Health and Disease. Information: AOCS Meetings Department, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

Training in Free Radical Methodologies, Sept. 23–27, 1996, CERLIB, University of Grenoble, Grenoble, France. Production, damages, repair: ESR, nitric oxide, DNA oxidation, DNA repair, apoptosis, chemical-trapping, lipoprotein oxidation, protein oxidation, cellular production and toxicity. Information: Arlette Alcaraz, Laboratoire de Biochimie C, CHU Grenoble BP 217, 38043 Grenoble Cédex 9, France (Phone: 33 76 76 57 54; Fax: 33 76 76 56 64; E-mail: CERLIB@ujf-grenoble.fr).

October 1996

Frontiers in Lipid and Lipoprotein Research: Basic Science, Analytical, Clinical, and Public Health Applications, Oct. 4–6, 1996, Stouffer Renaissance Hotel, Dallas, Texas. Sponsored by the Lipids and Lipoproteins Division of the American Association for Clinical Chemistry, the Centers for Disease Control and Prevention, and the American Heart Association. An integrated review of relevant recent advances in basic science, measurement technology, epidemiological and clinical research, and public health issues will be presented. This review will help define state-of-the-art techniques for understanding and preventing morbidity and mortality due to heart disease. Information: Paula M. Steiner, Medical Research Laboratories, 2 Tesseneer Dr., Highland Heights, KY 41076 (Phone: 606-781-8877, Extension 252; Fax: 606-781-9310; E-mail: lipf@cehehll.em.cdc.gov).

American College of Nutrition, 37th Annual Meeting, Oct. 11–13, 1996, San Francisco, California (Phone: 212-777-1037; Fax: 212-777-1103).

Certification Board for Nutrition Specialists, Oct. 11, 1996, San Francisco, California. Certifying examination for nutritionists and dieticians with advanced degrees. Information: CBNS, 1562 First Avenue, Box #258, New York, NY 10028 (Phone: 212-332-0774 (leave message); Fax: 212-777-1103).

Seminar: Interconversion of Polyunsaturated Fatty Acids, and Their Functions in Membranes, Oslo, Norway, Oct. 11–12, 1996. Information: Georg Lambertsen, Secretary, Lipidforum, Breistølen 1, N-5035, Bergen, Norway (Phone: 4755 + 32 89 56; Fax: 47 55 + 32 57 24).

"Confectionery Fats" Symposium, Belgrave Sq., London, Oct. 15–16, 1996, Oils and Fats Group—Society of the Chemical Industry, UK. Information: Secretariat, SCI, 14/15 Belgrave Sq., London.

November 1996
AOCS Conference on PUFA in Infant Nutrition: Consensus and Controversies, Nov. 7–9, 1996, Barcelona, Spain. Information: Susan E. Carlson (E-mail: scarlson@utmem1.utmem.edu); Robert A. Gibson (E-mail: rgibson@paed_nnu.fmc.flinders.edu.au); Berthold Koletzko (Fax: 49-89-5160-4733); or AOCS Meetings Department, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

March 1997
CERLIB 3rd Winter Research Conferences, Les Arcs 1800, France, March 9–14, 1997. Proteins and Free Radicals, from Radical Enzymes to Damages; Oxidative Effects of Radiations on Biomolecules and Cells; Oxidative Stress and Apoptosis. Information: Arlette Alcaraz, Laboratoire de Biochimie C, CHU Grenoble BP 217, 38043 Grenoble Cédex 9, France (Phone: 33 76 76 57 54; Fax: 33 76 76 56 64; E-mail: CERLIB@ujf-grenoble.fr).

April 1997
IV. International Dermatology Symposion Berlin, Sebaceous Gland and Its Disorders, Basic and Clinical Research, Clinical Entities and Treatment, April 11–13, 1997, University Medical Center Benjamin Franklin. Information: Docent Dr. Ch. C. Zouboulis, Department of Dermatology, University Medical Center Benjamin Franklin, The Free University of Berlin, Hindenburgdamm 20, D-12200 Berlin, Germany (Phone: 49-30-8445-2808; Fax: 49-30-8445-4262).

June 1997
The 19th Nordic Lipid Symposium, Ronneby, Sweden, June 15–18, 1997. Information: Georg Lambertsen, Secretary, Lipidforum, Breistølen 1, N-5035, Bergen, Norway (Phone: 47 55 + 32 89 56; Fax: 47 55 + 32 57 24).

July–August 1997
Fourth International Congress on Essential Fatty Acids and Eicosanoids, July 27–August 1, 1997, Edinburgh, Scotland. Information: Secretariat, c/o Meeting Makers, 50 George St., Glasgow G1 1QE, Scotland, United Kingdom.

Phosphatidate Phosphatases of Mammals, Yeast, and Higher Plants

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Department of Chemistry, University of Lethbridge, Lethbridge, Alberta T1K 3M4, Canada

ABSTRACT: Phosphatidate phosphatase (EC 3.1.3.4) catalyzes the hydrolysis of phosphatidate to yield *sn*-1,2-diacylglycerol and inorganic phosphate. In mammalian systems, forms of phosphatidate phosphatase involved in glycerolipid synthesis and signal transduction have been identified. Forms of the enzyme involved in signal transduction have been purified and partially characterized. In yeast, phosphatidate phosphatases associated with the endoplasmic reticulum and mitochondria have also been purified and partially characterized. Information on phosphatidate phosphatases from mammals and yeast is useful in characterizing the enzyme from plant systems. This review examines progress on the characterization of phosphatidate phosphatases from mammals, yeast, and higher plants. The purification and characterization of the phosphatidate phosphatase involved in glycerolipid synthesis in developing oilseeds may lead to the identification of the encoding gene. Increasing our understanding of the enzymes of lipid synthesis in developing seeds will aid in the development of biotechnological strategies for seed oil modification.
Lipids 31, 785–802 (1996).

Phosphatidate (PA) phosphatase (also called PA phosphohydrolase) (EC 3.1.3.4) catalyzes the hydrolysis of PA to generate *sn*-1,2-diacylglycerol (DG) and P_i (1). DG produced from the hydrolysis of PA is not only a direct precursor of triacylglycerol (TG), but also a substrate for the synthesis of membrane phospholipids (1,2). PA phosphatases involved in glycerolipid synthesis appear to exist in both soluble and particulate forms, with the distribution of these forms being affected by metabolic status (3,4). The intracellular translocation of PA phosphatase may help to regulate lipid metabolism (3). In mammalian systems, other forms of PA phosphatase have been identified which appear to be involved in signal transduction (5–7). The characterization of PA phosphatases from mammalian and yeast systems, however, is considerably more advanced than our understanding of forms of the enzyme in higher plant systems.

Developments in various areas of mammalian and microbial biochemistry often precede the development of our understanding of equivalent systems from plants. A major objective of this review is to integrate literature on eukaryotic PA phosphatases with the intention of providing a useful resource for plant lipid biochemists, physiologists, and biotechnologists involved in studying aspects of glycerolipid metabolism. We do anticipate, however, that the information in this review will also be useful to lipid biochemists in general. The properties of mammalian and yeast PA phosphatases are examined first, followed by a discussion of glycerolipid synthesis and PA phosphatases in higher plants.

MAMMALIAN PA PHOSPHATASES

PA phosphatase involved in glycerolipid metabolism. Extensive studies have been conducted on mammalian PA phosphatases involved in glycerolipid synthesis, but this review deals only with certain aspects of the literature. The microsomal form of PA phosphatase, which is mainly involved in glycerolipid metabolism, has been been referred to as PA phosphatase-1 (8,9). The enzyme translocates from the cytosol to the endoplasmic reticulum (ER) to become functionally active (8), has an absolute Mg^{2+} requirement (9), and is selectively inactivated by the thioreactive reagent, *N*-ethylmaleimide (NEM) (10). PA phosphatase-1 has not been purified to homogeneity from any mammalian source. PA phosphatase-1 activity, however, has been extensively characterized using several different tissues of mammalian origin (see Refs. 11–13 for reviews), including human adipose tissue (14).

Synthesis of the rat liver enzyme has been shown to be stimulated by glucagon, glucocorticoids, cAMP, and growth hormone (15–17); this action was antagonized by insulin (16). The level of PA phosphatase also seemed to be stabilized or maintained by glucagon, whereas insulin decreased the half-life of this activity (10,11). Humble and Berglund (18) have demonstrated that cytosolic PA phosphatase activity from rat liver could be influenced by a number of phospholipids when they were included with emulsions of PA. This suggested that

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Abbreviations: ACP, acyl carrier protein; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; CPT, cholinephosphotransferase; DG, *sn*-1,2-diacylglycerol; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; G3P, *sn*-glycerol-3-phosphate; LPA, lysophosphatidate; LPAAT, lysophosphatidate acyltransferase; LPCAT, acyl-CoA:lysophosphatidylcholine acyltransferase; MD, microspore-derived; NEM, N-ethylmaleimide; PA, phosphatidate; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine; PG, phosphatidylglycerol; pI, isoelectric point; TG, triacylglycerol.

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changes in the lipid environment may possibly play a role in modulating PA phosphatase activity *in vivo*. Berglund *et al.* (19) presented some evidence to support deactivation of PA phosphatase-1 by phosphorylation, but Butterwith *et al.* (20) were not able to confirm the findings.

The metabolic expression of PA phosphatase-1 activity is thought to be regulated by the ability of the cytosolic form to translocate to the ER, where its substrate is being generated (3,17,21). In rat liver, this intracellular movement seemed to be controlled mainly by the accumulation of fatty acids (22) and acyl-CoA esters (23) on the ER membranes. According to Hopewell *et al.* (21) and others (1,3,11), the binding of long-chain fatty acids and their CoA esters to the ER, as well as the accumulation of PA in this compartment, acts as a signal for more PA phosphatase to associate with the membranes, and thereby enhances the synthesis of glycerolipids, especially TG. It has been suggested that the translocation of PA phosphatase probably depends on the increased negative charge conferred to the ER membranes by these compounds (21), serving to activate PA phosphatase in order to match the rate of TG synthesis to the supply of fatty acids (3,17). Spermine also facilitated the association of PA phosphatase with the membranes of the ER (24), and its effect was synergistic with fatty acids in this respect (25). It appears that spermine may promote the association of hydrophobic proteins with lipids and membranes (21,26), possibly by forming complexes with acyl-CoA *in vivo* (24), and also by causing the aggregation of microsomal membranes *in vitro* (21). ADP, GDP, and CDP have been shown to activate rat liver phosphatase-1, whereas ATP was inhibitory (27). Short-term treatment with the amphipathic cationic drug, chlorpromazine, decreased the rate of TG and phosphatidylcholine (PC) biosynthesis in isolated rat hepatocytes, and decreased the rate of DG formation in a cell-free system of rat liver by displacing PA phosphatase from the microsomal membranes and also by preventing the soluble form of PA phosphatase from interacting with these membranes (21,28). Hopewell *et al.* (21) have indicated that chlorpromazine could oppose the intracellular translocation of PA phosphatase by donating a positive charge to the membranes. Additionally, there is some evidence that the regulation of PA phosphatase activity may be coordinated with that of CTP-phosphocholine cytidylyltransferase (EC 2.7.7.15), another translocatable enzyme involved in the glycerolipid metabolism of rat hepatocytes (29,30). Freeman and Mangiapane (31) have demonstrated that cytosolic rat liver PA phosphatase also translocates to the mitochondria and that mitochondrial PA could be converted to TG in rat hepatocytes (32).

The Mg^{2+}-dependent form of PA phosphatase from rat adipose tissue appears to have many characteristics in common with the enzyme from rat liver (13). The enzyme from rat adipose tissue, however, was not affected by the addition of ATP or ADP, suggesting that the enzymes from adipose tissue and liver may be regulated by different mechanisms, and may thus represent two different isoenzymes (33).

Besides the extensive studies on Mg^{2+}-dependent PA phosphatase from rat liver and adipose tissue, the enzyme has also been examined in a number of tissues from various mammalian species. For example, the enzyme has been studied in rod outer segments (34) and other subcellular fractions of bovine retina (35), as well as in pig (36) and mouse brain (37). PA phosphatase may play an important role in myocardial TG homeostasis, and the enzyme from rat heart shares many characteristics with the enzymes from rat liver and adipose tissue, including its stimulation by Mg^{2+} and oleate, and its regulation by subcellular translocation to the microsomal membranes in response to oleate (38). PA phosphatase from vertebrate lungs also functions in the production of pulmonary surfactant (12,39,40). The major component of pulmonary surfactant is the phospholipid, dipalmitoyl-PC (12), which is synthesized *via* the catalytic action of PA phosphatase in type II epithelial cells of the alveoli (39).

Further progress toward elucidating the mechanisms that control the PA phosphatase activity involved in glycerolipid metabolism, including its distribution in different cellular compartments, depends on the ability to purify and further characterize the Mg^{2+}-dependent enzyme. Butterwith *et al.* (15) have partially purified Mg^{2+}-dependent PA phosphatase from the cytosolic fraction of rat liver. Purification and characterization of Mg^{2+}-dependent PA phosphatases have been complicated by apparent aggregation of enzyme as observed during molecular sieve chromatography (41).

PA phosphatase involved in signal transduction. In addition to its pivotal role in glycerolipid metabolism, the PA phosphatase-catalyzed conversion of PA to DG is also important in signal transduction where DG serves as a second messenger (5–7). Recent studies also indicate that an important alternative route for the production of DG is through the breakdown of PC (8,9,42). The generation of DG can occur either directly as a result of phospholipase C activity or by the action of phospholipase D followed by PA phosphatase. The latter is an important route related to signal transduction DG liberation (43), and has been reported in rat liver (8,44), rat fibroblasts (5), Chinese hamster ovary cells (42), NG108-15 cells (6), porcine thymus (45), human neutrophils (42,46) and interleukin-1-stimulated human mesangial cells (47). PA may also be a potent second messenger involved in the modulation of protein kinase C, as well as several other processes (8,45). Therefore, it is most likely that PA phosphatase plays a role in destroying this signal while, at the same time, creating another through the formation of DG (42). The enzyme involved in cell signalling, which has been referred to as PA phosphatase-2 (5,8), is tightly bound to the plasma membrane (42), Mg^{2+}-independent (7,10), and insensitive to NEM (10,45).

PA phosphatases involved in signal transduction have been reported to have been purified from porcine thymus (45) and rat liver (48–50). Properties of highly purified preparations of these enzymes are summarized in Table 1. Solubilized PA phosphatase from porcine thymus membranes displayed a subunit molecular mass of 83 kDa based on SDS–polyacrylamide gel electrophoresis (PAGE) of the final enzyme preparation (45). A molecular mass of 218 kDa was determined by

TABLE 1
Properties of Phosphatidate (PA) Phosphatases Involved in Signal Transduction in Mammals[a]

PA phosphatase	Apparent molecular mass and charge	Kinetic and specificity properties	Activators and inhibitors	Other properties
Porcine thymus membranes (6,000–100,000 × *g*), Ref. 45	218 kDa (native) by gel filtration; 83 kDa (subunit) by SDS–PAGE (reducing and nonreducing)	K_m for bulk [PA] = 0.2 mM; K_m for surface [PA] = 0.3 mol%; specific activity (purified enzyme) = 15 μmol/min/mg protein; was inactive against LPA	Activators: Triton X-100, deoxycholate Inhibitors: Mn^{2+}, Ca^{2+}, Zn^{2+}, DG, sphingosine, chlorpromazine, NaF, propanolol	pH optimum 7–7.4; NEM-insensitive; Mg^{2+}-independent
Rat liver membranes (6,000–100,000 × *g*); PA phosphatase-2A (partially purified), Ref. 48	290–300 kDa (native) by gel filtration; 83, 54, and 34 kDa subunits detected by SDS–PAGE	Specific activity (partially purified enzyme) = 4.7 μmol/min/mg protein; accepted PA containing 6:0, 10:0, 14:0, 16:0, 18:0, 18:1, and 18:1-20:4; least active with 18:1–20:4-PA	Inhibitors: Mn^{2+}, Zn^{2+}, amphiphilic amines	NEM-insensitive
Rat liver membranes (6,000–100,000 × *g*); PA phosphatase-2B, Ref. 48	265 kDa (native) by gel filtration; 83 kDa (subunit) by SDS–PAGE (reducing and nonreducing)	K_m for bulk [PA] = 0.3 mM K_m for surface [PA] = 0.31 mol% V_{max} = 12.6 μmol/min/mg protein; specific activity (purified enzyme) = 10.7 μmol/min/mg protein accepted PA containing 6:0, 10:0, 14:0, 16:0, 18:0, 18:1, and 18:1–20:4; most active with 6:0-PA; dephosphorylated PA	Activators: Triton X-100, deoxycholate; Inhibitors: Mn^2, Zn^{2+}, Co^{2+}, DG, LPA, monoacylglycerol, spingosine, chlorpromazine, desipramine	pH optimum 6–7; NEM-insensitive; Mg^2-independent
Rat liver plasma membranes; cationic and anionic PA isolated; cationic form purified to homogeneity; Ref. 49	Cationic form 51 kDa by SDS–PAGE (reducing and non-reducing); anionic form about 53 kDa; pI cationic form = 9; pI anionic form < 4	Specific activities of cationic and anionic forms were 0.1 and 5.7 μmol/min/mg protein, respectively; both forms dephosphorylated LPA		NEM-insensitive; glycoproteins; the 2 forms appeared to be different sialated states of the same protein; immunochemical crossreactivity between anionic and cationic forms; anionic form a possible phosphoprotein
Rat liver membranes (34,500–235,000 × *g*), Ref. 50	186 kDa (native) derived from Stokes' radius and sedimentation coefficient; 31 kDa by SDS–PAGE			NEM-insensitive

[a]PAGE, polyacrylamide gel electrophoresis; LPA, lysophosphatidate; DG, *sn*-1,2-diacylglycerol; NEM, *N*-ethylmaleimide; pI, isoelectric point.

Superose 12 (Pharmacia Biotech, Uppsala, Sweden) gel filtration chromatography in the presence of 1% (wt/vol) Triton X-100 (Boehringer Mannheim GmbH, Mannheim, Germany), indicating a possible oligomeric structure for the enzyme. The enzyme was specific for PA, and was inhibited by the reaction product, DG.

Fleming and Yeaman (48) purified PA phosphatase-2 from a crude membrane fraction of rat liver. Gel filtration chromatography of the solubilized enzyme on Sephacryl S300 (Pharmacia) resulted in two major peaks of enzyme activity designated PA phosphatase-2A and 2B. The authors suggested that these may represent different isozymes of PA phosphatase-2. Each enzyme form was further purified independently by a series of chromatographic steps using buffers containing Triton X-100. Native PA phosphatase-2A eluted with an apparent molecular mass of 290-300 kDa during gel filtration chromatography on Superose 6. SDS–PAGE indicated that PA phosphatase-2A consisted of polypeptides with molecular masses of 83, 54, and 34 kDa. Futher purification failed to resolve these components. PA phosphatase-2B was purified to apparent homogene-

ity. Analytical gel filtration chromatography of PA phosphatase-2B using Superose 6 (Pharmacia) indicated an apparent molecular mass of 265 kDa. Analysis by SDS–PAGE resulted in detection of a polypeptide with molecular mass of 83 kDa, which was in agreement with results on porcine thymus PA phosphatase-2 (45). PA phosphatase-2A accepted a range of PA varying in fatty acyl chain length, but the enzyme was least active with arachidonyl-containing PA. PA phosphatase-2B also accepted a range of PA varying in acyl chain length, but it displayed a very strong preference for PA containing 6-carbon acyl chains. Fleming and Yeaman (48) further compared the properties of rat liver PA-phosphatase-2B to those of the porcine enzyme purified by Kanoh *et al.* (45). Both the porcine and rat liver enzymes were NEM-insensitive, and were inhibited by Zn^{2+}, Mn^{2+}, and amphiphilic amines. Neither enzyme was activated by fatty acids or phospholipids. The PA phosphatases from the two sources, however, differed in apparent native molecular mass. Rat liver PA phosphatase-2 also catalyzed the hydrolysis of lysophosphatidate (LPA) (48), the immediate precursor of PA (51).

In another report, Fleming and Yeaman (52) examined the subcellular distribution and some properties of NEM-sensitive and NEM-insensitive PA phosphatases in rat brain. NEM-sensitive PA phosphatase was associated with the cytosol and microsomes, whereas NEM-insensitive PA phosphatase was found in the synaptosomes. Translocation of cytosolic PA phosphatase was facilitated by oleate. NEM-sensitive PA phosphatase displayed increased specificity for PA with short acyl chains, whereas the NEM-insensitive form preferred both short acyl chains and long unsaturated acyl chains. NEM-insensitive PA phosphatase, however, was somewhat less effective in catalyzing the hydrolysis of arachidonyl-stearoyl-PA in comparison to dioleoyl-PA.

Waggoner *et al.* (49) purified a NEM-insensitive PA phosphatase from rat plasma membranes. The PA phosphatase was an integral membrane glycoprotein which appeared to be purified in two different sialated states. A cationic form [isoelectric point (pI) = 9] of the enzyme was purified to homogeneity, whereas an anionic form (pI < 4) was only partially purified. The apparent subunit molecular mass of the cationic form was 51 kDa based on SDS–PAGE. Antibodies raised against the cationic form were used to identify the anionic form in Western blots resulting from SDS–PAGE. The anionic form was about 2 kDa larger than the cationic form. Treatment of the anionic form with neuramindase generated a species with properties of the cationic form. Furthermore, treatment of both the cationic and anionic forms with *N*-glycanase F resulted in a 28 kDa band (based on SDS–PAGE and Western blot analysis). Treatment of anionic PA phosphatase with acid phosphatase altered the mobility of the enzyme based on analysis by SDS–PAGE and Western blotting, suggesting that anionic PA phosphatase was a phosphoprotein.

Recently, Siess and Hofstetter (50) identified a NEM-insensitive PA phosphatase following SDS–PAGE of enzyme which was partially purified from rat liver membranes. A denatured PA phosphatase with molecular mass of 31 kDa was detected on a nitrocellulose blot of the SDS-gel. Antiserum raised against partially purified enzyme was allowed to interact with the denatured enzyme followed by capture of native PA phosphatase from a solution containing active enzyme. The activity of the captured native enzyme was used to identify the position of the PA phosphatase subunit.

YEAST PA PHOSPHATASES

In *Saccharomyces cerevisiae*, the regulatory mechanisms and basic biosynthetic pathways involved in the formation of TG and other glycerolipids are very similar to those which occur in higher eukaryotes (53–55). Hosaka and Yamashita (55) demonstrated that PA phosphatase activity was associated with both membrane and cytosolic fractions from *S. cerevisiae*. They also partially purified and characterized the soluble form of PA phosphatase from this microorganism.

More recently, a microsomal PA phosphatase and another form of the enzyme present in both microsomes and mitochondria have been identified in *S. cerevisiae* (56,57). A 91-kDa form of the enzyme was purified to apparent homogeneity from the total membrane fraction of this yeast (58,59). Immunoblot analysis, using antibodies raised against this membrane-associated 91-kDa enzyme, showed that it was actually the proteolytic product of a 104-kDa form of microsomal PA phosphatase (57). Subsequently, using the same procedure employed to purify the 91-kDa protein, the 104-kDa form of the enzyme was purified from microsomes obtained from a strain of protease-deficient cells. The kinetic properties of the 104-kDa PA phosphatase were indistinguishable from the 91-kDa enzyme. Immunoblot analysis using antibodies specific for the 91-kDa enzyme also revealed the existence of a 45-kDa form of PA phosphatase, which was then purified to homogeneity from the mitochondrial fraction of *S. cerevisiae* (56,57). This was done by employing the same procedure initially used to purify the 91-kDa protein from the total membrane fraction. The 45-kDa form of the enzyme was shown to be associated with both the microsomal and mitochondrial fractions. The properties of purified PA phosphatases from *S. cerevisiae* are shown in Table 2.

The strictly microsomal, 91-kDa enzyme was monomeric, Mg^{2+}-dependent, stimulated by Triton X-100 and sensitive to thioreactive compounds (59). The apparent K_m for dipalmitoyl-PA and V_{max} were determined to be 50 μM and 30 μmol/min/mg, respectively (59). Kinetic analysis of purified 91-kDa PA phosphatase, using Triton X-100/PA mixed micelles, showed that enzyme activity was dependent on the bulk and surface concentrations of PA (60). This was consistent with a "surface dilution" kinetic scheme where PA phosphatase interacted with the mixed micelle surface before substrate binding and catalysis occurred. Based on kinetic constants, dicaproyl-PA was a better substrate than either dioleoyl-PA or dipalmitoyl-PA which, in relation to each other, were equally good substrates. The enzyme was inhibited by the phospholipid intermediate, cytidine diphospho-(CDP-)DG, and by the neutral lipids, DG and TG (59).

TABLE 2
Properties of Yeast PA Phosphatases[a]

PA phosphatase	Apparent molecular mass and charge	Kinetic and specificity properties	Activators and inhibitors	Other properties
Sacchromyces ceresisiae 91-kDa form purified from 1,500–100,000 × *g* fraction; Refs. 59, 60	93 kDa (native) by gel filtration; 91 kDa (subunit) by SDS–PAGE	K_m for bulk [PA] = 50 μM V_{max} = 30 μmol/min/mg protein; molecular activity = 2.7×10^3 min^{-1} specific activity (purified enzyme) = 29.5 μmol/min/mg protein; interfacial Michaelis constant (xK_m^B) for dioleoyl-PA = 0.14 mol fraction; specificity constants indicated that dicaproyl-PA was a better substrate than dipalmitoyl- or dioleoyl-PA (the latter two were equally good substrates); inactive against LPA; activation energy = 11.6 kcal/mol; assays conducted with Triton X-100/PA mixed micelles	Activators: Triton X-100 Mg^{2+}; Inhibitors: NEM, Hg^{2+}, *p*-chloromercuriphenyl-sulfonic acid, CDP-DG, DG, TG	pH optimum 7; degradation product of 104 kDa form, Ref. 136
S. cerevisiae 104-kDa form purified from microsomes of a protease-deficient strain; Refs. 57,61,62,64	104 kDa by SDS–PAGE	enzymological properties identical to the 91-kDa form	Activators: cardiolipin ($A_{0.5}^b$ = 1.9 mol%); CDP-DG ($A_{0.5}$ = 2.6 mol%); phosphatidylinositol ($A_{0.5}$ = 5.5 mol%) Inhibitors: phenylglyoxal (IC_{50} = 7.6 mM), propanolol (IC_{50} = 0.93 mM), ATP (IC_{50} = 1.3 mM), ADP (IC_{50} = 1.9 mM), CTP (IC_{50} = 1.1 mM), GTP (IC_{50} = 2.2 mM), UTP (IC_{50} = 1.3 mm), sphingosine (aK_i^c = 1.5 mol%), phytosphingosine (aK_i = 0.4 mol%), sphinganine (aK_i = 0.2 mol%),	pI = 6.0; located in microsomes
C. cerevisiae 45-kDa form purified from mitochondria; Refs. 57,61–64	45 kDa by SDS–PAGE	enzymological properties similar to 104-kDa form	Activators: Triton X-100; Mg^{2+}; anionic phospholipids Inhibitors: NEM, *p*-chloromercuriphenyl-sulfonic acid, phenylglyoxal (IC_{50} = 4.3 mM), propanolol (IC_{50} = 0.95 mM), nucleotides, sphingoid bases	pH optimum 6–7; pI = 8.3; located in mitochondria and microsomes; peptide fragments resulting from V8 proteolysis differ from the 104-kDa form; immunochemically related to 91-kDa and 104-kDa forms; induced by addition of inositol to growth medium; phosphorylated by cAMP-dependent protein kinase

[a]Abbreviations as in Table 1; TG, triacylglycerols. [b]$A_{0.5}$ = Concentration required for half-maximum activation (Ref. 62). [c]For aK_i, the term *a* takes in account an interaction factor for the inhibitor (Ref. 61).

The 45-kDa form of PA phosphatase was also Mg^{2+}-dependent and stimulated by Triton X-100 (56,57). Although the 45-kDa enzyme was associated with both the microsomal and mitochondrial fractions, the kinetic properties of this form of PA phosphatase were similar to those of the strictly microsomal enzyme when uniform mixed micelles containing Triton X-100 and PA were used. The 45-kDa and 91-kDa forms of the enzyme were also similar to each other with respect to their inhibition by various compounds and their temperature stability. The two forms of PA phosphatase, however, differed in their pI, and in the peptide fragments generated as a result of V8 proteolysis and cyanogen bromide cleavage.

A number of possible regulatory mechanisms for controlling lipid metabolism at the level of PA phosphatase have been investigated in *S. cerevisiae*. Evidence for possible *in vivo* regulation by certain sphingoid bases (61) and anionic phospholipids (62) has been presented. Wu *et al.* (61) demonstrated that the dependence of PA phosphatase activity on PA was cooperative in the absence or presence of sphingosine, phytosphingosine, and sphinganine, and that these compounds inhibited purified preparations of both the 104-kDa and 45-kDa forms of PA phosphatase in a dose-dependent manner. They also found that these sphingoid bases were parabolic competitive inhibitors of PA phosphatase activity, indicating that more than one inhibitor molecule contributed to the exclusion of PA from the enzyme, and that both phytosphingosine and sphinganine were inhibitory at near endogenous concentrations. More recently, both the 104-kDa and 45-kDa forms of PA phosphatase were shown to be activated by the anionic phospholipids cardiolipin, CDP-DG, and phosphatidylinositol (62). Enzyme activation followed positive cooperative kinetics and activation appeared to be caused by anionic phospholipids decreasing the K_m for PA. The anionic phospholipids were stimulatory near their endogenous concentrations.

Quinlan *et al.* (63) have shown that the 45-kDa enzyme from *S. cerevisiae* is probably regulated by a mechanism involving reversible cAMP-dependent protein phosphorylation. For example, cAMP-dependent protein kinase catalyzed the phosphorylation of pure 45-kDa PA phosphatase at a serine residue, which resulted in a 2.4-fold stimulation of PA phosphatase activity. Dephosphorylation of the pure 45-kDa enzyme was catalyzed by alkaline phosphatase, and resulted in a 1.3-fold inhibition of PA phosphatase activity. Purified 104-kDa PA phosphatase, however, was not phosphorylated by cAMP-dependent protein kinase, strongly suggesting that the two yeast enzymes were differentially regulated. These findings were corroborated by *in vivo* phosphorylation studies using *S. cerevisiae* mutants (*bcy 1* and *bcy 2*) defective in cAMP-dependent protein kinase activity.

Wu and Carman (64) examined the possible regulation of PA phosphatase from *S. cerevisiae* by nucleotides. Both the 104- and 45-kDa enzymes were inhibited by a number of nucleotides, with ATP and CTP being the most effective inhibitors. Further experiments with the 104-kDa enzyme indicated that the mechanism of inhibition was due to chelation of the Mg^{2+}, which serves as the enzyme's cofactor. The inhibitory effect of ATP was considered physiologically relevant as the inhibitor constant for ATP was in the range of its cellular concentration.

At the genetic level, certain factors regulate the expression of PA phosphatase in *S. cerevisiae*. It is not yet clear what the relative contributions of the 45-kDa and 104-kDa forms of PA phosphatase are to the overall TG and phospholipid synthesis rates in *S. cerevisiae*, but it has been found that PA phosphatase activity increases when wild-type cells are supplemented with inositol (65) and when cells enter the stationary phase of growth (53,65). CDP-DG synthase (CTP:phosphatidate cytidylyltransferase, EC 2.7.7.41) catalyzes the generation of CDP-DG and pyrophosphate from CTP and PA, and provides phosphatidyl moieties for the synthesis of major phospholipids (54). The 45-kDa form of PA phosphatase has been shown to be induced in the presence of inositol (57), and the activity of CDP-DG synthase was repressed (54). The activity of the 104-kDa PA phosphatase, however, was not affected by treatment with inositol (57). When yeast cells entered the stationary phase of growth, both the 104-kDa and the 45-kDa PA phosphatase were induced (57), and CDP-DG synthase activity was again repressed (54). The increase in PA phosphatase activity in response to inositol supplementation was correlated with an increase in phospholipid content at the expense of TG (57). In contrast, the increase in PA phosphatase activity observed during the stationary phase of growth correlated with an increase in TG content at the expense of phospholipid (53). Thus, in *S. cerevisiae*, the flux of acyl chains through DG and CDP-DG appears to be controlled by the relative activities of both forms of PA phosphatase, as well as by CDP-DG synthase.

GLYCEROLIPID SYNTHESIS IN HIGHER PLANTS

As indicated earlier in this review, one of our goals was to provide useful information for plant scientists studying PA phosphatases. In order to clarify the context in which plant PA phosphatases function, we briefly review TG biosynthesis as it relates to phospholipid metabolism in higher plants. The reader is also referred to other detailed reviews of plant lipid biosynthesis (66,67) and biotechnology (68,69).

The major pathway for the formation of TG was first elucidated using animal tissues through the work of Kennedy (51,70,71), Kornberg and Pricer (72), and others (73,74). Early evidence for Kennedy pathway activity in plants was based on experiments by Barron and Stumpf (75), which demonstrated that a particulate fraction from avocado mesocarp was capable of generating radiolabeled TG from [^{14}C]-*sn*-glycerol-3-phosphate (G3P). The formation of TG from G3P and fatty acyl-CoA precursors is known as the G3P or Kennedy pathway (51). The G3P dehydrogenase-catalyzed reduction of dihydroxyacetone phosphate, derived from cytosolic glycolysis and the pentose phosphate pathway, gives rise to G3P (76,77). In some cases, G3P may also be formed by the direct phosphorylation of glycerol, which is catalyzed by glycerol kinase (77,78).

De novo biosynthesis of fatty acids occurs within the plastids (79). Starting with acetyl moiety, the process involves several enzymes and a sequence of repeating reactions which results in the formation of fatty acids up to the level of 16:0, 18:0, and 18:1 (80,81). These fatty acids are generated as thioesters linked to acyl carrier protein (ACP), and are either used directly for plastidial glycero- and galacto-lipid biosynthesis or end up as fatty acyl-CoA in the cytoplasm (82). An acyl-ACP hydrolase may catalyze the hydrolysis of acyl-ACP liberating fatty acids for movement across the plastidial envelope (76). Fatty acyl-CoA are then generated by the action of an acyl-CoA synthetase, which is associated with the outer membrane of the plastid (76).

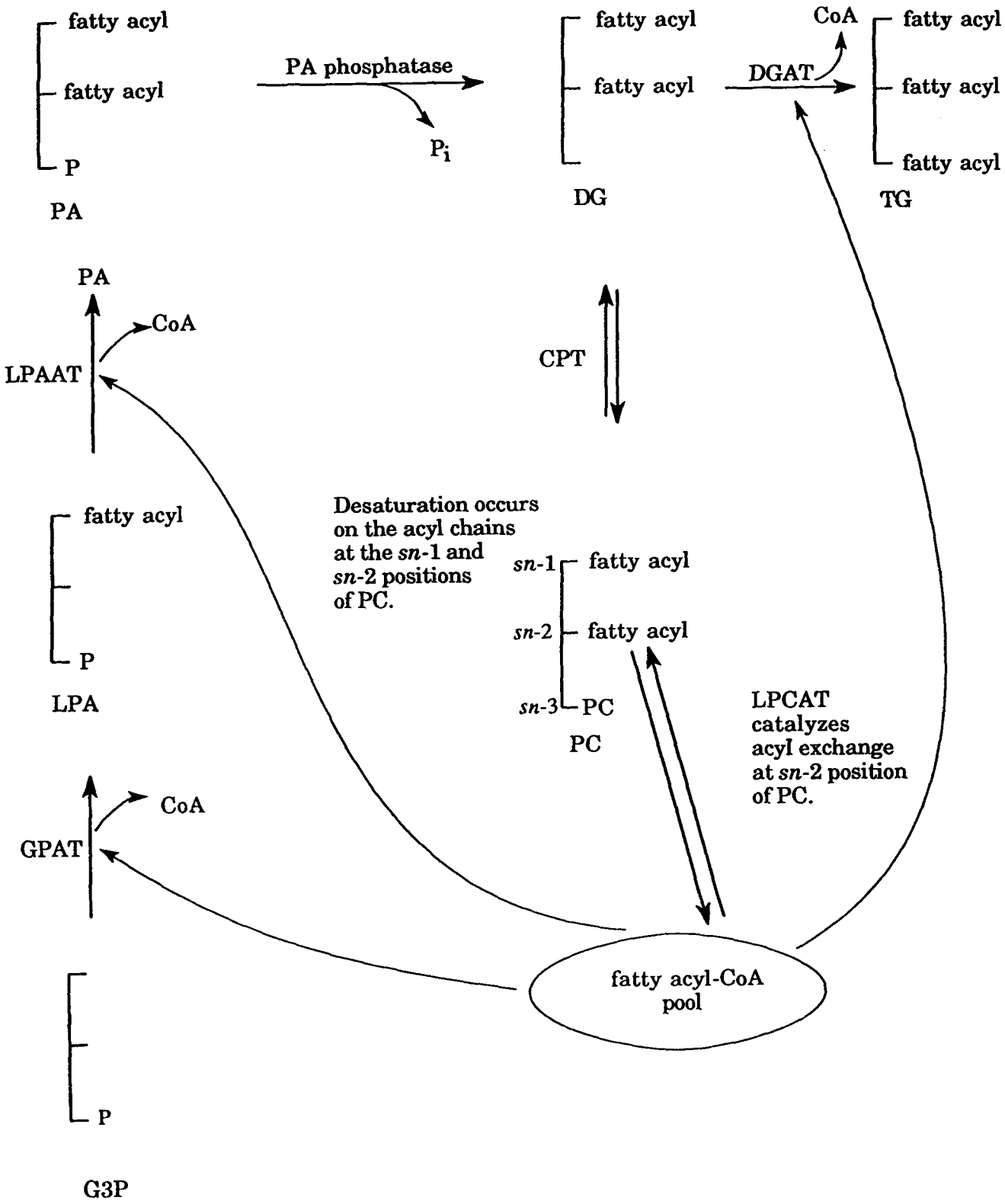
The Kennedy pathway enzymes (51,76), which are responsible for catalyzing TG bioassembly, are primarily associated with the ER. Strong evidence for this was provided by Stobart *et al.* (83), who demonstrated that microsomes from safflower seeds were capable of TG synthesis at rates comparable to the maximum *in vivo* (84) rate of TG deposition. Reactions involved in TG bioassembly in developing oilseeds are outlined in Scheme 1. The first enzyme of the Kennedy pathway, glycerophosphate acyltransferase (EC 2.3.1.15), catalyzes the acylation of G3P at the *sn*-1 position to form LPA. The second acyltransferase, lysophosphatidate acyltransferase (LPAAT; EC 2.3.1.51), is responsible for the *sn*-2 acylation of LPA to yield PA. It is the acyl specificity and selectivity properties of these two enzymes that govern the nonrandom distribution of fatty acid species at the *sn*-1 and *sn*-2 positions of PA and DG and the other glycerolipids which are subsequently derived from them (85,86). Recently, cDNA clones encoding LPAAT have been isolated and characterized for maize (87), *Limnanthes douglasii* (88), and immature coconut *(Cocos nusifera)* endosperm (89). PA phosphatase catalyzes the cleavage of PA to release P_i and the key intermediate in TG biosynthesis, DG. Diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) is the enzyme responsible for the ultimate step in the biosynthesis of TG, and catalyzes the acylation of the *sn*-3 position of DG previously occupied by the P_i group. DGAT is partially responsible for regulating the fatty acid composition TG as it catalyzes the only step in the Kennedy pathway which is committed to TG biosynthesis (76). Identification of the remaining genes encoding the Kennedy pathway enzymes of the ER could contribute to the improvement of oilseed crops, but molecular genetic studies have been impeded by the difficulties experienced in the solubilization and purification of these enzymes (90–93).

The biosynthesis of membrane lipids share the same steps with the Kennedy pathway up to, and including, the point where DG is formed (78,94). These activities are localized in several compartments of the cell and are often collectively referred to as the Kornberg–Pricer pathway (95,96). In higher plants, a portion of DG derived from PC, synthesized in the ER, is directed to chloroplasts (80,82). Phosphatidylglycerol (PG) is the only product of plastidial lipid synthesis in many angiosperm families which have been designated 18:3 plants (67,80,82,97). In these 18:3 plants, other chloroplast lipids are synthesized using DG derived from the ER. The 18:3 plants contain galactolipids with substantial amounts of α-linolenic acid (67,82,97). In comparison, in some primitive angiosperms, such as *Arabidopsis* and spinach, both plastidial and extraplastidial DG contribute equally to the synthesis of galactolipids and sulfolipids (67,82,98). These latter species are designated as 16:3 plants because their leaf galactolipids are enriched in hexadecatrienoic acid (16:3n-3). For more detailed information on the pool of DG as it relates to synthesis of sulfolipids and galactolipids in plants, the reader is referred to Mudd and Kleppinger-Sparace (97) and Joyard *et al.* (99).

The acyl-CoA pool represents a complex collection of fatty acyl moieties in the developing seed (82). Desaturation-catalyzed reactions utilize PC as a substrate for the introduction of additional double bonds in the acyl chains at the *sn*-1 and 2 positions (76). The production of TG enriched in polyunsaturated fatty acids has been attributed to an acyl-exchange mechanism for releasing the desaturated products from the phospholipid back to the acyl-CoA pool (100) (see Scheme 1; adapted from Refs. 76 and 101). Acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT; EC 2.3.1.23) may catalyze the exchange of fatty acyl groups between 18:1-CoA and the polyunsaturated 18-carbon fatty acyl groups esterified to the *sn*-2 position of PC (76,101). The reaction is known to occur in reverse to facilitate entry of 18:2 and 18:3 into the acyl-CoA pool (101). In addition, LPCAT catalyzes the acylation of the *sn*-2 position of lysophosphatidylcholine to form PC (101). The combined effects of desaturation on PC and acyl exchange at the *sn*-2 position of PC lead to an acyl-CoA pool enriched in 18:2 and 18:3 moieties, which can then become substrates for the acyltransferases of the Kennedy pathway (76,78).

The production of TG enriched with polyunsaturated fatty acids has also been attributed to the flux of DG through the PC pool (76,80). DG generated through hydrolysis of PA by PA phosphatase in the Kennedy pathway is converted to PC by a freely reversible reaction catalyzed by CDP-choline 1,2-diacylglycerol cholinephosphotransferase (CPT) (EC 2.7.8.2) (2,25) (see Scheme 1). The acyl group at the *sn*-2 position of PC is capable of being rapidly desaturated as described above, but in this case the DG skeleton of PC is returned to the DG pool and converted into TG. Using microsomal preparations of cotyledons from developing safflower seeds, Stobart and Stymne (2) were able to demonstrate the rapid exchange of DG with PC, and Griffiths *et al.* (102) showed that DG equilibrated with the PC pool when the glycerol backbone, and associated acyl groups, flowed from PA to TG. Thus, the levels of DG used for TG biosynthesis are controlled through the activities of both PA phosphatase and CPT. Recently, a soybean (*Glycine max* L.) cDNA was was used to complement a CPT-deficient mutant of yeast (103).

The coordination of the Kennedy pathway enzymes with CPT, LPCAT, and oleate and linoleate desaturases is responsible for the synthesis of TG having 18:2 and 18:3 at the *sn*-2 position of the glycerol backbone (76,101). However, attempting to determine the relative contributions of CPT and



SCHEME 1

PA phosphatase in customizing the DG pool is difficult because PC and TG synthesis both appear to occur within the ER (82). In some developing seeds of oilseed plants the situation is further complicated by the fact that certain fatty acyl moieties are largely or completely excluded from the membrane lipids (78,104,105). Using homogenates obtained from the developing seeds of safflower, *Crambe abysinnica*, and *Cuphea wrightii*, Battey and Ohlrogge (106) demonstrated

that exogenously supplied medium- and long-chain fatty acids were targeted to TG and not to phospholipids.

Erucic acid (22:1) is one example of an unusual fatty acid that is channeled to reserve lipid. Several varieties of *Brassica napus* and *B. rapa* (syn. *campestris*) produce seed oil characterized by a large amount of erucic acid. Seed oils high in 22:1 are desirable as starting material for the production of high temperature lubricants, nylon, and plasticizers (107), and thus there is considerable interest in developing genetically manipulated *B. napus* which produces trierucoylglycerol (108–111). In high erucic acid rapeseed lines, this long-chain monounsaturated fatty acid is associated with the *sn*-1 and *sn*-3 positions of the TG molecules (112), but is essentially excluded from membrane constituents (106). Using *B. napus* microspore-derived (MD) embryos of the high erucic acid cultivar, Reston, Taylor *et al.* (108) demonstrated that [^{14}C]-erucoyl moieties from [^{14}C]-22:1-CoA were rapidly incorporated into TG, but were virtually excluded from complex polar lipids. Erucic acid is synthesized by the elongation of oleoyl-CoA (113,114) and the process does not involve, to any extent, a PC intermediate (80). Elongation occurs *via* two successive condensations in the presence of malonyl-CoA and reductant, resulting in 2-carbon extensions from 18:1-CoA *via* 20:1-CoA to 22:1-CoA, with retention of the relative ω-9-position of the double bond introduced during oleate synthesis in the plastid (113). Low erucic acid cultivars appear to simply lack elongase activity (75,115). Many *Brassica* species do not contain significant amounts of erucic acid at the *sn*-2 position (109). Thus, the theoretical breeding limit for 22:1 in *B. napus* seed oils was 66 mol% (110,111,115). Recently, a cDNA encoding LPAAT from developing seeds of meadowfoam *(Limnanthes alba alba)* was expressed in developing seeds of transgenic high-erucic acid *B. napus* (116). The transgenic seeds produced some trierucin, but the total erucic acid content was similar to the control seed. This work, however, constitutes a breakthrough in the genetic manipulation of acyltransferases to generate seeds oils with desired fatty acid compostion.

In other cases unusual fatty acids have been shown to be transiently incorporated into PC prior to their eventual accumulation as TG. In developing castor bean *(Ricinus communis)*, synthesis of ricinoleic acid (18:1-OH) was shown to occur by hydroxlyation of the oleoyl moiety while it was attached to the *sn*-2 position of PC (117). Oleate was released through the action of a phospholipase A_2 and was activated to ricinoleoyl-CoA which, in turn, served as substrate for the acyltransferases of the Kennedy pathway. In addition, PC containing oxidized acyl groups was more efficiently degraded by phospholipase then PC containing nonoxidized acyl groups (118). Thus, phospholipases may contribute to the exclusion of fatty acids containing oxidized acyl groups from membrane lipids (117,118). This may also serve as a mechanism for membrane repair (117). Ståhl *et al.* (119) have indicated that phospholipases specific for unusual acyl groups might also degrade PA. However, PA was a less efficient substrate than PC and represented a relatively small component of the total phospholipid pool in maturing oilseeds.

Petroselinic acid, the Δ-6 *cis* isomer of oleic acid, is another example of an unusual fatty acid (120). Cahoon and Ohlrogge (120) conducted time-course labeling and pulse-chase studies with [1-^{14}C]acetate using developing endosperm from the Umbelliferae species coriander (*Coriandrum sativum* L.) and carrot (*Daucus carota* L.). Radiolabeled petroselinic acid initially entered PC but accumulated in TG at a later time. Unlike ricinoleic acid, however, petroselinic acid was not further modified on PC.

Recently, Vogel and Browse (121) investigated the relative roles of CPT and DGAT in channeling unusual fatty acids into TG during seed maturation. In experiments with various oilseeds, CPT could not differentiate between DG molecular species containing 18:1 and those containing 10:0, 22:1, and 18:1-OH. Thus, CPT did not appear to be involved in the exclusion of unusual fatty acids from PC. DGAT from castor bean endosperm, however, displayed an enhanced preference for DG species containing two ricinoleic acid residues. CPT from the same plant did not exclude this fatty acid residue. Thus, it appeared that castor bean DGAT was involved in channeling ricinoleic acid into TG. The investigators suggested that there may be a number of mechanisms contributing to the exclusion of unusual fatty acids from membranes. These mechanisms may include the action of phospholipases specific for unusual acyl groups, the molecular species specificity of DGAT, and spatial separation of membrane lipid synthesis and TG bioassembly within the ER.

PLANT PA PHOSPHATASES

Although they have not been as well studied as the comparable enzymes in either mammalian tissues or yeast, PA phosphatase activities have been partially characterized in the plastids, microsomes, and soluble fractions of a number of plant tissues (for examples see Refs. 122–126). Kates (127) first showed that the 1,800–15,000 × *g* sediment from spinach leaf homogenate could catalyze the hydrolysis of PA to form P_i, but he generally employed PC as the substrate and followed the liberation of both choline and P_i. The formation of P_i was slower than that of choline and was preceded by a lag period, suggesting that PC was converted to PA and subsequently hydrolyzed to DG and P_i. In the same study, Kates (127) showed that the P_i could have arisen from either, or both, of the PC degradation products, PA or G3P, but not from LPA.

Leaf PA phosphatases. Königs and Heinz (128) investigated the subcellular distribution of PA phosphatase in broadbean *(Vicia faba)* leaves and partially characterized the enzyme. When the crude subcellular fractions obtained by the differential centrifugation of tissue homogenate were assayed at pH 5.6, PA phosphatase activity was almost exclusively associated with a 40,000 × *g* supernatant and a 600–15,000 × *g* sediment. About 80% of the total activity of the homogenate was found in the 40,000 × *g* supernatant. The cytosolic and particulate enzymes exhibited pH optima of 5.1 and 5.6, respectively, and both forms of PA phosphatase activity were

inhibited by Ca^{2+} ions. The enzyme in the 600–15,000 × *g* sediment was solubilized by sonciating in the presence Triton X-100.

The effect of herbicides on PA phosphatase and other enzymes involved in glycerolipid metabolism has been investigated in the leaves of groundnut *(Arachis hypogaea)*. Rajasekharan and Sastry (129,130) have shown that, although other enzyme activities involved in lipid metabolism were inhibited, both the plastidial and microsomal PA phosphatase activities from leaf disks of groundnut were insensitive to phenoxy, thiocarbamate, urea, and uracil herbicides.

The majority of research on leaf PA phosphatases has involved investigations of the plastidial enzyme. Studies have been conducted with nongreen starch-containing plastids from sycamore and cauliflower (125), as well as the chloroplasts of spinach (131,132) and pea (133). In plastids, DG is a source of acyl chains for galactolipids and sulfolipid (96,99). PA synthesized in plastids is metabolized into either DG or CDP-DG, and it is probably one of the major regulatory points for controlling the biosynthesis of plastidial membrane lipids and the final proportions of PG, galactolipids (monogalactosyldiacylglycerol and digalactosyldiacylglycerol), and sulfolipid (sulfoquinovosyldiacylglycerol) (96,99). The formation of DG occurs in the plastids as the result of a membrane-bound PA phosphatase (131,134), which is exclusively located on the inner envelope membrane (124,133). In contrast to plastids from 16:3 plants, those from 18:3 plants have a rather low PA phosphatase activity and cannot deliver DG fast enough to sustain maximal rates of galactolipid synthesis (99,135,136). These results explain why 18:3 plants contain only small amounts of galactolipids and sulfoquinovosyldiacylglycerol with 16-carbon fatty acids at the *sn*-2 position, but contain PG, which is synthesized from PA *via* CDP-DG. It is not known, however, whether the reduced level of plastidial PA phosphatase activity in 18:3 plants is due to a lower species-specific expression of the gene coding for the enzyme, or to the presence of regulatory molecules which control the activity of PA phosphatase in this compartment (99).

The PA phosphatase activity associated with plant plastids appears to exhibit a number of biochemical properties (95,132) which are clearly different from those described for the PA phosphatase activities involved in glycerolipid metabolism in the various cell fractions obtained from mammals and yeast. Excluding the cell-signalling form of PA phosphatase associated with the plasma membrane, all the PA phosphatase activities from yeast (57,59) and mammals (10,15,38,137) were Mg^{2+}-dependent, regardless of the cell fraction serving as the source of enzyme activity. Additionally, the PA phosphatase activities from yeast (55,56,58) and mammals (13,36) usually exhibited pH optima which were within one pH unit of neutrality. In marked contrast, the plastidial enzyme appeared not to need divalent cations, which, in fact, seemed to inhibit the enzyme, and plastidial PA phosphatase exhibited maximal activity at pH 9.0. This alkaline pH optimum appears to be physiologically relevant because Sauer and Heise (138) have shown that light-activated fatty acid synthesis in spinach chloroplasts was dependent on stromal pH, and that the optimum pH for fatty acid synthesis was close to 8.5. Therefore, it appears that plastidial glycerolipid metabolism may be linked to the biosynthesis of fatty acids, at least partly, through the effects of pH in this subcellular compartment. Feedback inhibition by DG probably plays a important role in the regulation of plastidial PA phosphatase which, in turn, would influence the rate of galactolipid and sulfolipid biosynthesis (95). Using isolated intact chloroplasts and envelope membranes from thermolysin-treated chloroplasts, Malherbe *et al.* (132) have shown that DG is a powerful competitive inhibitor of the spinach chloroplast enzyme. They determined an apparent K_i of 70 μM using *sn*-1,2-diolein, whereas the apparent K_m for *sn*-1,2-dioleoyl-PA was as high as 600 μM. When incubations were conducted in the presence of different molecular species of DG, at the same concentration as *sn*-1,2-diolein, PA phosphatase inhibition occurred in the same concentration range, but *sn*-1,3-dipalmitin had almost no inhibitory effect. It appears that the *in vivo* steady state activity of plastidial PA phosphatase is sensitive to the DG/PA molar ratio, as increasing this ratio resulted in a logarithmic increase in PA phosphatase inhibition. Because the apparent K_i value for DG was much lower than the apparent K_m for PA, it has been suggested that the rate of DG formation is closely related to the rate of its utilization by the plastidial enzymes involved in galactolipid and sulfolipid synthesis (96). Thus, feedback inhibition of PA phosphatase by DG would most likely lead to an accumulation of PA and would favor PG synthesis in the plastid envelope (132).

Recently, Malherbe *et al.* (139) reported the solubilization of PA phosphatase from spinach chloroplast envelope membranes using 3-[(3-cholamidopropy)-dimethylammonio]propanesulfonate (CHAPS). In the presence of CHAPS, 1 mg PG per mL of reaction mixture stimulated PA phosphatase activity by 2–4-fold, while the same concentration of PC inhibited the enzyme. Solubilized plastidial PA phosphatase was inhibited by Mg^{2+} and a number of other metal ions, and DG was a strong inhibitor. Solubilized PA phosphatase was fractionated by hydroxyapaptite chromatography into two major peaks of activity and one apparently minor component. Most of the enzyme activity passed through the column during sample application and washing. The specific activity of the unbound fraction was similar to that of the solubilized enzyme. The final peak of activity, which required the highest concentration of potassium phosphate for elution, represented about 15% of the initial activity applied to the column. This fraction exhibited a specific activity four to five times greater than that of the solubilized enzyme.

PA phosphatases from germinating seeds. Most of the research on PA phosphatases in seeds has involved extraplastidial forms of the enzyme. The cotyledons of fully imbibed or germinating mung beans *(Vigna radiata)*, which have been shown to contain at least two distinct PA phosphatase activities (140), are one example. In this case, an enzyme exhibiting a pH optimum of 7.5 was located on the ER, and a second

PA phosphatase with a pH optimum of 5.0 was found associated with the protein bodies. The PA phosphatase activity exhibiting the acidic pH optimum was very easily released into the soluble fraction during subcellular fractionation and could be partially separated from general acid phosphatase activity by chromatography. The apparent molecular mass of the acidic PA phosphatase was estimated to be about 37 kDa by gel filtration chromatography.

Moore *et al.* (141) examined the characteristics of PA phosphatase activity in subcellular fractions obtained from the endosperm of germinating seeds of castor bean. When enzyme assays were conducted at pH 7.4, PA phosphatase activity was found mainly in the microsomes with some activity in the soluble fraction. Later, when subcellular fractions were assayed under more acidic conditions (142), it was found that the cytosolic fraction contained most of the PA phosphatase activity. The ER-associated enzyme described by Moore *et al.* (141) was shown not to hydrolyze glucose 6-phosphate or *o*-carboxyphenyl phosphate, suggesting that the PA phosphatase activity in this fraction was not due to a nonspecific phosphatase. *p*-Nitrophenyl phosphate, however, was not tested as a possible substrate. The PA phosphatase activity associated with the endosperm from germinating castor beans is most likely not involved in TG biosynthesis, but instead probably plays a role in membrane formation and other processes involved in germination and seedling growth.

It has been demonstrated that microsomal PA phosphatase activity is involved in the degradation of membrane phospholipids in the cotyledons of germinating seeds of *Phaseolus vulgaris* (143). The activity of the enzyme was stimulated by Ca^{2+} and was calmodulin-dependent.

PA phosphatases from developing seeds. In developing oilseeds, the DG formed by the PA phosphatase-catalyzed hydrolysis of PA is not only a precursor of TG but is also a substrate used for the synthesis of membranes (144). Using the developing seeds from groundnut *(A. hypogaea)*, Sukumar and Sastry (145) showed that PA phosphatase was associated with both the microsomal and mitochondrial fractions. In contrast to plastidial PA phosphatase of leaf tissue (139), the enzyme activity from maturing groundnut seeds depended upon the inclusion of $MgCl_2$ in the homogenizing buffer. PA phosphatase activity was not found in the cytosolic fraction, and only a negligible amount (<1%) was associated with the fat fraction containing the oil bodies. Both the microsomal and mitochondrial PA phosphatase activities showed a significant increase in specific activity between 20 and 30 d after fertilization, with maximal specific activity occurring 30 d after fertilization.

The dephosphorylation of PA during TG formation by microsomal fractions obtained from the cotyledons of developing safflower seeds was demonstrated by Stymne *et al.* (100) using three cultivars differing in seed oil fatty acid composition. These cultivars included a high oleate (75% oleate, 16% linoleate) variety, a very high linoleate (6% oleate, 88% linoleate) variety, and a cultivar known as Gila (16% oleate, 75% linoleate). At the same time, an examination of the ability of different molecular species of acyl-CoA to be incorporated into phospholipids and acylglycerols in Gila and high oleate varieties gave some information about the substrate specificities of microsomal PA phosphatases (100). The results from this study strongly suggested that microsomal PA phosphatase did not show a marked substrate specificity. Ichihara (126) showed that the microsomal PA phosphatase in maturing seeds of safflower was specific and selective for unsaturated forms of PA, and when the different forms of PA were ranked, the order of selectivity was similar to that of the specificity. Additionally, in agreement with Stymne *et al.* (100), Ichihara (126) concluded that the broad selectivity for unsaturated PA species (*sn*-1,2-diunsaturated-PA and *sn*-1-saturated, 2-unsaturated-PA) indicated that the PA phosphatase reaction probably has little effect on the fatty acid composition of TG in the oil obtained from safflower. Furthermore, compared with the safflower microsomal enzyme, the chloroplast PA phosphatase of spinach leaves showed a broader specificity (126), which agreed with the selectivity profile indicated by labeling patterns of PA and DG synthesized from [^{14}C]-acetate previously demonstrated in spinach chloroplasts (94).

Griffiths *et al.* (102) showed that microsomal PA phosphatase from the developing seeds of safflower has an absolute Mg^{2+} requirement. This was confirmed by Ichihara *et al.* (122), who demonstrated that other divalent cations, such as Ca^{2+} and Mn^{2+}, could not be substituted for Mg^{2+}. Additionally, it was shown that the microsomal enzyme exhibited a pH optimum of 6.75 and was inhibited by the polyamines, spermidine and spermine, but not by putrescine, which is the diamine precursor of spermidine and spermine (122). It has been suggested that polyamines, which are ubiquitous in plant tissues, may affect the synthesis of TG in maturing oilseeds by modulating the enzyme activity of each step in the Kennedy pathway (122). The soluble form of the enzyme from developing safflower seeds has not been characterized, but has been suggested to be a functionally inactive cytosolic reservoir of the microsomal form of the enzyme (4). Thus, as in the mammalian system (3), it appears that in maturing safflower seeds PA phosphatase translocates between the cytosol and the ER in response to the fatty acid level in the cytosol (4). This may explain some of the conflicting reports resulting from studies using plant tissues, with some investigators believing PA phosphatase to be a membrane-associated enzyme and some claiming that it is soluble (123,144,146).

The combined and relative activities of the microsomal and cytosolic forms of PA phosphatase varied as function of safflower cotyledon development (4). Combined PA phosphatase activity in the tissue was highest during the period of rapid oil accumulation (13–15 d postanthesis), when the majority of the PA phosphatase was membrane-bound. At the initial and last stages of seed development, when TG synthesis was not significant, the majority of the enzyme was soluble. Additionally, palmitic, stearic and oleic acids, which are the fatty acid products formed in the plastids, caused the

translocation of PA phosphatase from the cytosol to the microsomes. Laurate and linoleate, which are not products of plastidial fatty acid biosynthesis, showed no effect. The intracellular translocation induced by oleate was reversed by its removal. The metabolically significant thioester, oleoyl-CoA, did not convert the soluble form of the enzyme into the membrane-bound form. The cytosolic form of PA phosphatase from safflower seeds could be induced to translocate to the microsomes prepared from the sunflower seeds, but not to the microsomes prepared from the seeds of oilseed rape or soybean.

PA has been shown to be a major product when microsomes obtained from developing safflower cotyledons were incubated with the Kennedy pathway precursors, fatty acyl-CoA and G3P (2). This suggested that PA phosphatase activity may have a rate-limiting role in TG biosynthesis. In contrast, Ichihara *et al.* (122) have indicated that the amount of PA phosphatase activity in developing safflower seeds was enough to account for the amount of TG being formed *in vivo*, and that DGAT may catalyze the rate-limiting step in TG biosynthesis. Previously, Ichihara *et al.* (147) reported that DGAT had the lowest specific activity of all the Kennedy pathway enzymes in particulate fractions obtained from developing safflower seed, suggesting a rate-limiting role for DGAT. More recently, Perry and Harwood (148,149) have shown that DG accumulates during the period of rapid TG accumulation in the zygotic embryos of *B. napus*, suggesting that DGAT exerts significant flux control over the Kennedy pathway.

The appearance of TG biosynthetic enzyme activities in developing oilseeds are coordinated with TG biosynthesis. Studies with developing seeds of *B. napus* have shown that the activities of the three acyltransferases of the Kennedy pathway increased greatly during the rapid phase of oil accumulation (150). Weselake *et al.* (151) have confirmed this observation for the developmental profile of DGAT activity during maturation of *B. napus* L. cv Westar seed. In developing safflower seeds, both PA phosphatase (4) and DGAT (151) activities increased during the rapid phase of oil accumulation and declined markedly as lipid levels reached a plateau. A similar relationship was reported earlier for microsomal PA phosphatase and DGAT activities in relation to oil accumulation in developing groundnut seeds (145).

A more detailed understanding of the biochemical and genetic regulation of the PA phosphatase involved in TG biosynthesis in developing seeds will be realized through further characterization and purification of the enzyme. One of the greatest difficulties in working with PA phosphatases, however, is reliably measuring the enzyme activity in homogenates and crude subcellular fractions. Plant biochemists should be aware of the enzyme assay problems that have been encountered in investigations with mammalian systems. In studies with microsomes from rat liver, Sturton and Brindley (152), have indicated that measurement of P_i overestimated PA phosphatase because phosphate was also produced from dephosphorylation of G3P derived from the deacylation of PA by phospholipases. In contrast, Ide and Nakazawa (153) found that measurement of P_i was more reliable in estimating PA phosphatase activity than monitoring the appearance of DG when using cytosolic and microsomal fractions from rat liver. In their studies, DG was rapidly deacylated by reactions catalyzed by acyl hydrolases. Thus, when embarking on the study of PA phosphatase in crude fractions, in addition to running the appropriate controls, it is also important to initially assay the enzyme using different procedures (10) and to examine the products generated when the enzyme assay is complete (153).

Microspore-derived embryos of *B. napus* accumulate TG in a fashion similar to zygotic embryos (154,155), and have proven useful in the characterization, solubilization, and partial purification of TG biosynthetic enzymes (91,93,108,111, 151,155,156). They are easy to manipulate in culture and large numbers of embryos at specific developmental stages can be readily obtained. Recent studies of TG biosynthesis and enzyme characterization have also been conducted using an embryogenic MD cell suspension culture of *B. napus* L. cv Jet Neuf (91,151). The cell suspension culture contained 3–4% TG on a dry weight basis under normal culture conditions (151). The TG content and the activity of DGAT, however, could be manipulated by altering the sucrose concentration of the growth medium (unpublished observations, Weselake, R.J., Byers, S.D., Davoren, J.M., Laroche, A., Hodges, D.M., Pomeroy, M.K., and Furukawa-Stoffer, T.L.). Therefore, this system may be potentially useful for identifying cDNA encoding enzymes of TG bioassembly through analyses of gene expression in cells grown under different sucrose concentrations.

Kocsis *et al.* (93) characterized PA phosphatase activity in soluble and microsomal fractions from developing seeds of *B. napus*. Soluble PA phosphatase activity may have been attributable to nonspecific acid phosphatase activity with a pH optimum of about 5, whereas microsomes displayed optimal activity in the range of pH 6–7. PA phosphatase was solubilized from microsomes of MD embryos using Tween 20. Gel filtration chromatography of the solubilized enzyme revealed a minimum molecular mass of about 40 kDa. The solubilized enzyme had a preference for dioleoyl-PA and palmitoyl-PA over distearoyl-PA. More recently, the PA phosphatase from microsomes of MD embryos of *B. napus* was found to be composed of both NEM-sensitive and NEM-insensitive activity, and part of the overall activity was also Mg^{2+}-dependent (unpublished observations, Furukawa-Stoffer, T.L., Weselake, R.J., and Pomeroy, M.K.). Therefore, it is possible that the membrane fractions from MD embryos of *B. napus* contained PA phosphatases involved in both glycerolipid biosynthesis and signal transduction.

In future research, it might be worthwhile to attempt photoaffinity labelling of the PA phosphatase involved in glycerolipid biosynthesis in developing seeds using a radiolabeled azido-derivative of PA. Photoaffinity labeling of the PA phosphatase polypeptide may serve as probe to facilitate enzyme purification. Ideally, labeling would have to be carried out

under conditions which minimized or eliminated the hydrolysis of azido-PA. Recently, the photoreactive substrate analogue, 12-[(4-azidosalicyl)amino]dodecanoyl-CoA, was found to be potentially useful in the purification of DGAT (157). This agent inhibited microsomal DGAT activity from MD embryos of *B. napus* and was used to label polypeptides in solubilized fractions containing DGAT activity. Alternatively, the genes encoding PA phosphatase and DGAT involved in glycerolipid biosynthesis in developing seeds may be identified through molecular genetic strategies. Indeed, mutants of lipid synthesis in plants have proven useful in the identification of genes encoding various enzymes involved in lipid metabolism (90).

A more complete picture of the biochemical regulation of PA phosphatase and DGAT in developing seeds awaits the identification of possible allosteric effectors which may modulate the activities of these enzymes. These enzymes may also be subject to phosphorylation/dephosphorylation mechanisms which are, in turn, dependent on other cellular conditions. As indicated previously, the 45-kDa form of yeast PA phosphatase is activated by phosphorylation catalyzed by a cAMP-dependent protein kinase (63). There is also compelling evidence that mammalian DGAT is regulated by reversible protein phosphorylation (158–160). It is possible that PA phosphatase and DGAT in the ER of the developing seed may interchange roles as rate-limiting enzymes depending on the metabolic status of their surroundings. The possible effects of oleate on promoting the interaction of solubilized PA phosphatase with microsomes may provide insights into the biochemical regulation of PA phosphatase and also contribute to the development of a procedure for reconstitution of PA phosphatase with membrane components.

Knowledge of the biochemical properties of PA phosphatase and DGAT may facilitate effective biotechnological strategies for increasing seed oil content and/or modifying the fatty acid composition of seed oil. Increasing PA phosphatase and/or DGAT production may lead to increased seed oil content. In addition, seed oil composition might be altered by modifying the genes encoding these enzymes. One might envision the development of a transgenic plant producing seeds which express a form of PA phosphatase which preferentially accepts specific species of PA. It may also be possible to modify potential regulatory sites on PA phosphatase so it responds to its metabolic environment in an altered fashion.

Role of PA phosphatase in phospholipid degradation and turnover. Besides its important role in glycerolipid synthesis, PA phosphatase is also involved in phospholipid degradation and phospholipid turnover in general. As previously discussed, microsomal PA phosphatase has been shown to be involved in the degradation of membrane phospholipids in germinating seeds (143). A similar mechanism also seems to be operative in the microsomal membranes from senescing carnation flowers (161,162). Additionally, the decrease in microsomal PA phosphatase activity, relative to the other enzymes involved in phospholipid degradation, has been implicated in chilling injury in tomato fruit (163). It appears that the chilling injury occurring during low temperature storage of tomato fruit may be the result of the accumulation of PA and other lipid degradation intermediates in the membranes due to the differential effects of reduced temperature on the activity of PA phosphatase and other lipid-degrading enzymes.

Highly purified preparations of NEM-insensitive PA phosphatases involved in signal transduction have been characterized in mammalian systems. Substantial progress has also been made in the characterization of purified forms of PA phosphatase from *S. cerevisiae*. It is clear from the literature that the study of PA phosphatases from mammals and yeast has had a significant impact on the design of experiments used in the characterization of plant PA phosphatases. No form of PA phosphatase, however, has been highly purified from any plant source. Identification of the gene encoding PA phosphatase involved in glycerolipid synthesis in developing oilseeds could contribute to the development of biotechnological strategies for increasing seed oil content and modifying the fatty acid composition of seed oil. Increasing seed oil content would be of great economic benefit. Modification of seed oil composition could contribute to product diversification, including production of nutritionally desirable oils and oils for the manufacturing industry.

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Specificity of Two Lipoxygenases from Rice: Unusual Regiospecificity of a Lipoxygenase Isoenzyme

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ABSTRACT: The regio- and stereospecificity of two lipoxygenases from rice were investigated using arachidonic acid as the substrate. Rice seed lipoxygenase-2 (RSL-2) catalyzed oxygenation of arachidonic acid into a mixture of 5(*S*)-hydroperoxy-6,8,11,14-eicosatetraenoic acid [5(*S*)-HPETE] and 15(*S*)-hydroperoxy-5,8,11,13-eicosatetraenoic acid [15(*S*)-HPETE]. In addition, two double dioxygenase products, 5(*S*),15(*S*)-dihydroperoxy-6,8,11,13-eicosatetraenoic acid and 8(*S*),15(*S*)-dihydroperoxy-5,9,11,13-eicosatetraenoic acid, were obtained in a lower yield. The regiospecificity of the RSL-2-catalyzed oxygenation was pH-dependent. Thus, incubation at pH 6.7 led to the formation of 5(*S*)-HPETE and 15(*S*)-HPETE in a ratio of 52:48, and incubation at pH 9.8 strongly suppressed production of 5(*S*)-HPETE and led to formation of 5(*S*)-HPETE and 15(*S*)-HPETE in a ratio of 3:97. A pH-dependent orientation of arachidonic acid at the active site is proposed to explain these findings. Rice leaf pathogen-inducible lipoxygenase [Peng, Y.-L., Shirano, Y., Ohta, H., Hibino, T., Tanaka, K., and Shibata, D. (1994) *J. Biol. Chem. 269*, 3755–3761] catalyzed oxygenation of arachidonic acid into a single hydroperoxide isomer of high optical purity, i.e., 15(*S*)-HPETE (99.5% *S*).

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Presently, intense research is devoted to understand the molecular mechanisms involved in plant protection to microbial infection. Apart from being of biological interest, the agricultural and environmental protection aspects of such work is of obvious importance. In rice plants, a number of oxylipins, notably epoxy, hydroxy, epoxy-hydroxy, and trihydroxy derivatives of linoleic and α-linolenic acids, are produced in response to attack by the rice blast fungus (1–5). Levels of lipoxygenase and of enzyme(s) catalyzing degradation of hydroperoxides increase in rice leaves following infection with an incompatible race of the rice blast fungus (6,7). The nature of the hydroperoxide-decomposing enzyme activity in rice is not well established; however, it seems likely that at least part of this activity is due to the enzyme peroxygenase (8), which catalyzes reduction of fatty acid hydroperoxides with concomitant formation of epoxy- and epoxy-hydroxy fatty acids (9–11). Importantly, a number of oxygenated fatty acids produced from linoleic and α-linolenic acids in rice show antifungal activity. Thus, germination and germ tube elongation of spores of the rice blast fungus are inhibited by low concentrations (2–40 ppm) of the oxygenated fatty acids (12–14). Furthermore, when rice plants are grown in a nutrient solution supplemented with C_{18} epoxy acids and subsequently inoculated with rice blast fungus, the number of lesions appearing on leaves is dramatically reduced compared to that observed in the nonsupplemented controls (14).

Oxylipins derived from fatty acids other than linoleic and α-linolenic acids also can be important in plant defensive reactions against pathogens. For example, the fungus *Phytophthora infestans* elicits necrosis and accumulation of antimicrobial compounds in potato tubers. The causative agent in this process appears to be arachidonic acid, which is present in the fungal mycelia (but not in potato) (15). Arachidonic acid supplied from the fungus is converted into hydroperoxide derivatives by potato tuber lipoxygenase. The hydroperoxides and/or secondary metabolites formed from them appear to be of critical importance as elicitors, because the response reaction in the tuber tissue is blocked by lipoxygenase inhibitors (16,17).

The present paper is concerned with the structure and stereochemistry of products formed from arachidonic acid upon incubation with two rice lipoxygenases, i.e., rice seed lipoxygenase-2 (RSL-2) (18) and rice leaf pathogen-inducible lipoxygenase (RLIL) (19). Comparison of the specificities of oxygenations of arachidonic acid and linoleic acid is of general biochemical interest. Furthermore, although arachidonic acid is not present in rice, characterization of its lipoxygenase products is of potential biological importance in view of the possibility that arachidonic acid may be supplied to the rice plant by attacking fungi and insects.

EXPERIMENTAL PROCEDURES

Fatty acids and fatty acid hydro(pero)xides. Arachidonic and linoleic acids were purchased from Nu-Chek-Prep (Elysian,

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Abbreviations: 9(*S*)-H(P)OD, 9(*S*)-hydro(pero)xy-10(*E*),12(*Z*)-octadecadienoic acid; 13(*S*)-H(P)OD, 13(*S*)-hydro(pero)xy-9(*Z*),11(*E*)-octadecadienoic acid; 5(*S*)-H(P)ETE, 5(*S*)-hydro(pero)xy-6(*E*),8(*Z*),11(*Z*),14(*Z*)-eicosatetraenoic acid; 15(*S*)-H(P)ETE, 15(*S*)-hydro(pero)xy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid; 5(*S*),15(*S*)-diH(P)ETE, 5(*S*),15(*S*)-dihydro(pero)xy-6(*E*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid; 8(*S*),15(*S*)-diH(P)ETE, 8(*S*),15(*S*)-dihydro(pero)xy-5(*Z*),9(*E*),11(*Z*),13(*E*)-eicosatetraenoic acid; GC/MS, gas chromatography/mass spectrometry; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; Me_3Si, trimethylsilyl; PAP, *N*-(propionoxyacyl)-L-phenylalanine-methyl ester; RLIL, rice leaf pathogen-inducible lipoxygenase; RSL-2, rice seed lipoxygenase-2; SP, straight-phase; TLC, thin-layer chromatography; UV, ultraviolet.

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MN). [1-^{14}C]Arachidonic and [1-^{14}C]linoleic acids were obtained from DuPont NEN (Boston, MA). 9(*S*)-Hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid [9(*S*)-HPOD] [enantiomeric composition: 9(*S*)/9(*R*), 98:2] and 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid [13(*S*)-HPOD] [enantiomeric composition: 13(*S*)/13(*R*), 99:1] were prepared by incubations of linoleic acid with tomato lipoxygenase (20) and soybean lipoxygenase (21), respectively. 9(*S*)-HOD and 13(*S*)-HOD were prepared from the hydroperoxides by reduction with sodium borohydride in methanol. 9(*R,S*)-Hydroxy 10(*E*), 12(*E*)-octadecadienoic acid and 13(*R,S*)-hydroxy-9(*E*),11(*E*)-octadecadienoic acid were prepared by autoxidation of linoleic acid followed by reduction of the resulting hydroperoxides and isolation by high-performance liquid chromatography (HPLC) (22). 15(*S*)-Hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid [15(*S*)-HPETE] and 5(*S*)-hydroperoxy-6(*E*), 8(*Z*),14(*Z*)-eicosatetraenoic acid [5(*S*)-HPETE] were prepared by incubation of arachidonic acid with soybean lipoxygenase (21) and potato lipoxygenase (23), respectively. Aliquots of the hydroperoxides were treated with sodium borohydride to afford 15(*S*)-HETE and 5(*S*)-HETE. The methyl esters of 5(*R,S*)-HETE, 8(*R,S*)-HETE, 9(*R,S*)-HETE, 11(*R,S*)-HETE, 12(*R,S*)-HETE, and 15(*R,S*)-HETE were obtained by autoxidation of arachidonic acid followed by reduction, esterification, and isolation by straight-phase (SP) HPLC using a solvent system composed of 2-propanol/hexane (1.3:98.7, vol/vol).

Chemical methods. Catalytic hydrogenation (24), oxidative ozonolysis (25), and steric analysis of hydroxy acids (26) were performed as described.

Chromatographic and instrumental methods. Thin-layer chromatography (TLC) was carried out with precoated plates (Kieselgel 60, 0.25 mm) from E. Merck (Darmstadt, Germany). Material was located by spraying with 2′,7′-dichlorofluorescein and viewing under ultraviolet (UV) light. Radioactivity on TLC plates was determined with a Berthold Dünnschichtscanner II (Berthold Analytical Instruments, Wildbad, Germany) interfaced with a Macintosh SE/30. SP-HPLC was performed using a column of Nucleosil 50-5 (200 × 4.6 mm) purchased from Macherey-Nagel (Düren, Germany). The solvent system used for isolation of monohydroxy esters was 2-propanol/hexane (1.3:98.7, vol/vol; flow rate, 1 mL/min), whereas 2-propanol/hexane (2.5:97.5, vol/vol; flow rate, 1.5 mL/min) was used for isolation of methyl dihydroxy- and dihydroperoxyeicosatetraenoates. The absorbancy and radioactivity of HPLC effluents were determined on-line using a Spectromonitor III (Laboratory Data Control, Riviera Beach, FL) and a liquid scintillation counter (IN/US Systems, Tampa, FL), respectively. Gas–liquid chromatography (GLC) was performed with a Hewlett-Packard (Avondale, PA) model 5890 gas chromatograph equipped with a methyl silicone capillary column (length, 25 m; film thickness, 0.33 μm). Helium at a flow rate of 25 cm/s was used as the carrier gas. Gas chromatography/mass spectrometry (GC/MS) was carried out with a Hewlett-Packard model 5970B mass selective detector connected to a Hewlett-Packard model 5890 gas chromatograph. UV spectra were recorded with a Hitachi (Tokyo, Japan) model U-2000 UV/VIS spectrophotometer. Infrared spectrometry was carried out using a Perkin-Elmer (Norwalk, CT) model 1650 FT-IR spectrophotometer. Radioactivity was determined with a Packard Tri-Carb model 4450 liquid scintillation counter (Packard Instruments, Downer's Grove, IL).

Expression of lipoxygenases. RSL-2 and RLIL were expressed in *Escherichia coli* BL21 (DE3) carrying pET3/RSL-2 (18) or pET3/RLIL (19) essentially as described. Briefly, *E. coli* cells were grown at 37°C to an A_{600} of 1.0–1.5 in 100 mL of Luria-Bertani medium (1% Trypton, 0.5% yeast extract and 1% sodium chloride) containing 50 μg/mL ampicillin. Isopropyl-thio-β-D-galactoside (0.4 mM) was added, and the culture was shaken at 15°C for 16 h. The cells were collected by centrifugation at 9300 × *g* for 15 min at +3°C and washed by resuspension in 100 mL of buffer A (10 mM sodium phosphate pH 6.8, 10% glycerol, 0.1% Tween 20, and 0.1 mM EDTA) followed by centrifugation. The pellet of washed cells was suspended in 50 mL of buffer A and sonicated at 0°C (4 periods of 15 s). Particulate material was removed by centrifugation, and the supernatant was used as the enzyme source. Protein was determined according to Bradford (27).

Lipoxygenase incubations. For assay of lipoxygenase activity, aliquots of the enzyme preparations obtained as described previously were added to 3 mL of 0.1 M potassium phosphate buffer pH 6.7, and arachidonic or linoleic acids (final concentrations, 50 μM) were added in 3 μL of ethanol. The A_{235} was followed vs. time. Preparative incubations were carried out at 23°C by stirring arachidonic or linoleic acids (100 or 300 μM as indicated) in 0.1 M potassium phosphate buffer, pH 6.7 (50–200 mL), in the presence of enzyme preparation. The reaction product was extracted with diethyl ether, and aliquots were esterified and analyzed by radio-TLC or radio-HPLC before and after reduction with sodium borohydride. The remaining part was reduced by treatment with sodium borohydride and esterified. Isolation of mono- and dihydroxyesters was carried out by SiO_2 open column chromatography followed by SP-HPLC.

RESULTS

Enzyme activity of preparations of RSL-2 and RLIL was assayed spectrophotometrically using arachidonic and linoleic acids as the substrates. As seen in Figure 1A, linoleic acid was a better substrate than arachidonic acid for RSL-2. A significant lag phase (approximately 1 min) was apparent with both acids. The progress of the oxygenations ceased at about 40% conversion (arachidonic acid) and 75–80% conversion (linoleic acid). This was probably due to inactivation of the enzyme because further conversion of the acids could be achieved by addition of a second aliquot of enzyme. The specific activities of RSL-2 in oxygenations of arachidonic and linoleic acids (measured at the point of maximum slope of the progress curves; ε = 26,000) were 0.27 μmol/min • mg protein and 0.61 μmol/min • mg protein, respectively. Arachidonic and linoleic acids were oxygenated at approximately the same rates upon incubation with RLIL (Fig. 1B). In this case, the lag phase was less pro-

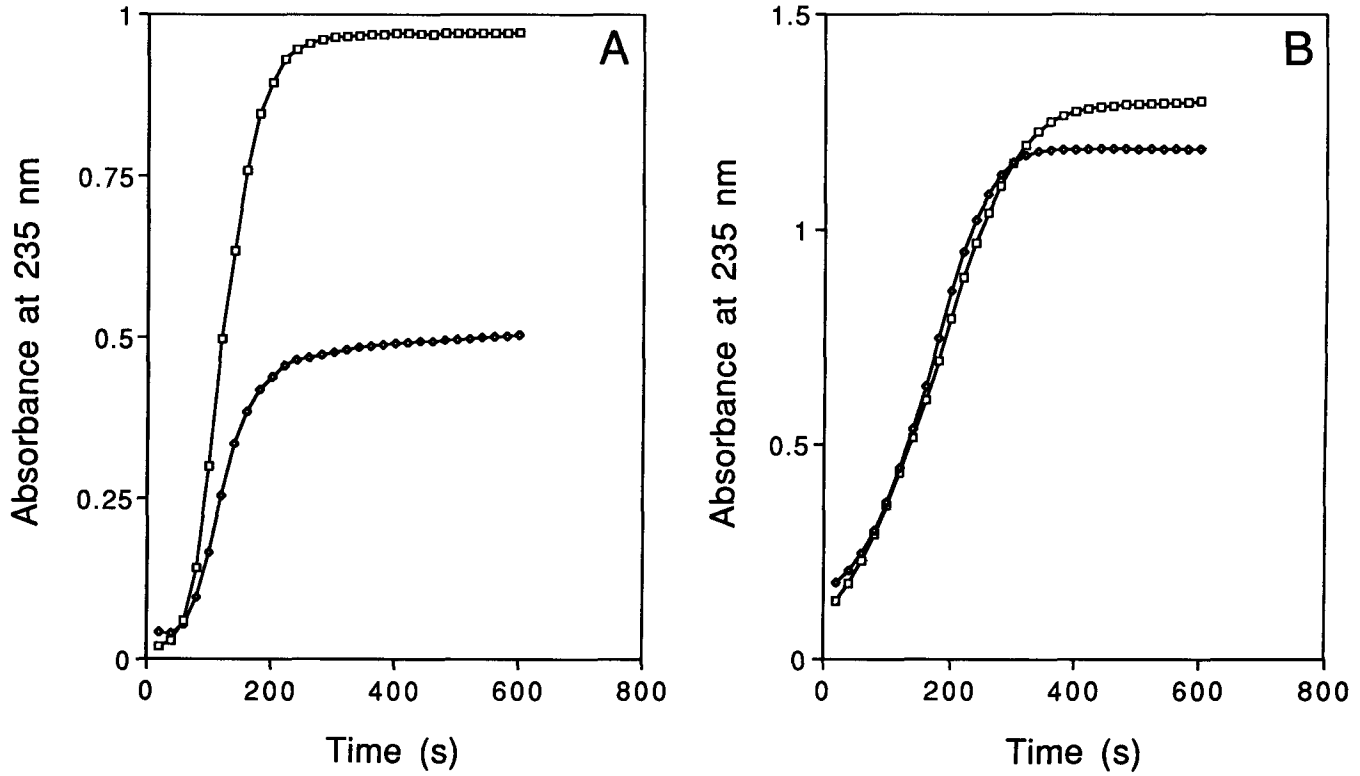


FIG. 1. Oxygenation of arachidonic and linoleic acids by rice seed lipoxygenase-2 (RSL-2) (A) and rice leaf pathogen-inducible lipoxygenase (RLIL) (B). The substrates (50 μM) were added dissolved in 3 μL of ethanol to preparations of RSL-2 (0.12 mg of protein in 3.2 mL of 0.1 M potassium phosphate buffer pH 6.7) or RLIL (0.03 mg of protein in 3.05 mL of 0.1 M potassium phosphate buffer pH 6.7). The absorbancy at 235 nm was followed vs. time. □, Linoleic acid; ◇, arachidonic acid.

nounced, and essentially complete conversions were observed. The specific activities of RLIL were 1.31 and 1.16 μmol/min • mg protein in oxygenations of arachidonic and linoleic acids, respectively.

Products formed from linoleic acid upon incubation with RSL-2. [1-^{14}C]Linoleic acid (300 μM) in 0.1 M potassium phosphate buffer pH 6.7 (100 mL) was stirred with enzyme preparation (3 mg of protein) for 20 min at 23°C. Material obtained by extraction with diethyl ether was subjected to SiO_2 open column chromatography. The column was eluted successively with diethyl ether/hexane (7:93, vol/vol; fatty acid fraction; less than 1% of the radioactivity applied), diethyl ether/hexane (20:80, vol/vol; hydroperoxide fraction; 86% of the radioactivity), and methanol (polar material; 14% of the radioactivity). Material present in the hydroperoxide fraction was reduced by treatment with sodium borohydride in methanol, esterified by treatment with diazomethane, and subjected to SP-HPLC. A main peak of material (retention volume, 7.6–8.1 mL) showing strong UV absorbance at 234 nm appeared. This compound was identified as the methyl ester of 13(*S*)-HOD by the following analyses (*cf.* Refs. 9,22): (i) cochromatography with authentic material on TLC and SP-HPLC; (ii) UV-spectrometry (λ_{max} = 234 nm, solvent ethanol); (iii) GC/MS analysis of the Me_3Si derivative using the same derivative of authentic material as reference; (iv) catalytic hydrogenation followed by GC/MS analysis of the Me_3Si derivative; and (v) oxidative ozonolysis performed on the propionyl derivative followed by coupling of the resulting 2-propionoxyheptanoic acid with L-phenylalanine methyl ester, and steric analysis of the resulting *N*-(propionoxyacyl)-L-phenylalanine-methyl ester (PAP) derivative by GLC (*cf.* Ref. 26). This last-mentioned analysis showed that the enantiomeric composition was 91% 13(*S*)-HOD and 9% 13(*R*)-HOD. In addition to 13(*S*)-HOD, three other hydroxyoctadecadienoates were observed upon SP-HPLC analysis. The identity of these compounds with the methyl esters of 9(*R,S*)-HOD [9(*R*)/9(*S*), 50:50; retention volume, 9.5–9.8 mL], 13-hydroxy-9(*E*),11(*E*)-octadecadienoic acid (8.7–9.0 mL), and 9-hydroxy-10(*E*),12(*E*)-octadecadienoic acid (10.4–10.8 mL) was established as described earlier using the respective authentic compounds as references. The composition of the hydroperoxide fraction obtained following incubation of linoleic acid with RSL-2 was: 13(*S*)-HPOD (87%), 9(*R,S*)-HPOD (8%), 9-hydroperoxy-10(*E*),12(*E*)-octadecadienoic acid (3%), and 13-hydroperoxy-9(*E*),11(*E*)-octadecadienoic acid (2%). A similar pattern of products was observed in incubations using a lower concentration (100 μM) of linoleic acid. That RSL-2 preferentially introduces oxygen at C_{13} when linoleic acid is used as the substrate has been previously reported (18).

Products formed from linoleic acid upon incubation with RLIL. [1-^{14}C]Linoleic acid (100 μM) was stirred with enzyme preparation (3 mg of protein) in 0.1 M potassium phosphate

buffer pH 6.7 (200 mL) at 23°C for 15 min. SiO_2 open column chromatography performed as described previously gave a hydroperoxide fraction that accounted for >90% of the radioactivity applied. Reduction with sodium borohydride followed by treatment with diazomethane and isolation by SP-HPLC afforded the methyl ester of 13-HOD (98%) and the methyl ester of 9-HOD (2%). Identification of the two compounds was performed by chemical and spectroscopical methods as described for the incubation of linoleic acid with RSL-2. The absolute configuration of the 13-HOD was found to be "*S*", i.e., 99.2% "*S*" and 0.8% "*R*." The configuration of the 9-HOD was not determined.

Products formed from arachidonic acid upon incubation with RSL-2. [1-^{14}C]Arachidonic acid (100 μM) in 0.1 M potassium phosphate buffer pH 6.7 (45 mL) was stirred with RSL-2 at 23°C. Three additions of enzyme preparation were made, i.e., 5 mL (3 mg of protein) at times 0, 10, and 20 min. After a stirring time of 30 min, the product was extracted with diethyl ether, reduced by treatment with sodium borohydride, and esterified. SiO_2 open column chromatography showed four main peaks of radioactivity, i.e., the methyl ester of arachidonic acid not converted [17%; eluted with diethyl ether/hexane (10:90, vol/vol)], methyl monohydroxyeicosatetraenoates [41%; eluted with diethyl ether/hexane (35:65, vol/vol)], methyl dihydroxyeicosatetraenoates (28%; eluted with ethyl acetate), as well as very polar material which was not further characterized (14%; eluted with methanol).

The methyl monohydroxyeicosatetraenoates were subjected to SP-HPLC (Fig. 2A). As seen, the methyl esters of 15-HETE (retention volume, 10.0–10.7 mL of effluent) and 5-HETE (22.4–23.7 mL of effluent) were obtained as the major compounds. In addition, small amounts of the methyl esters of 12-HETE, 11-HETE, 9-HETE, and 8-HETE, as well as the δ-lactone form of 5-HETE, appeared. The identities of the compounds were established by their retention volumes on SP-HPLC, by UV spectrometry, and by GC/MS analysis of the Me_3Si derivatives using the authentic compounds as references. In the cases of 5-HETE and 15-HETE, the absolute con-

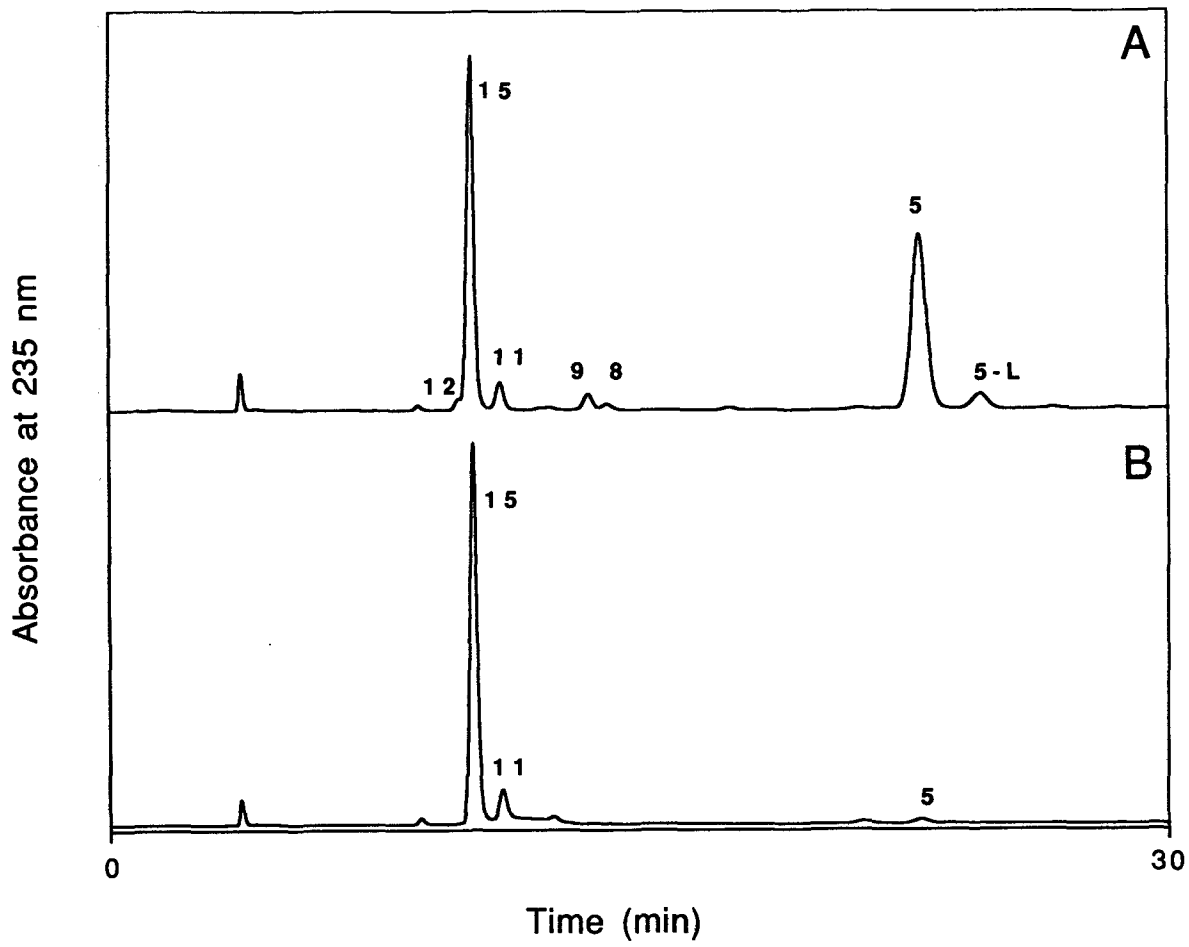


FIG. 2. High-performance liquid chromatography (HPLC) analysis of methyl monohydroxyeicosatetraenoates obtained following incubation of arachidonic acid with RSL-2 at pH 6.7 (A) and at pH 9.8 (B). The products of the incubations were reduced with sodium borohydride, esterified, and subjected to SiO_2 open column chromatography. Material present in the hydroxyester fraction (elution with diethyl ether/hexane, 35:65, vol/vol) was analyzed by straight-phase HPLC using a solvent system of 2-propanol/hexane (1.3:98.7, vol/vol) at a flow rate of 1 mL/min. 5-L, δ-lactone form of 5-HETE; 5, methyl ester of 5-HETE; 8, methyl ester of 8-HETE; 9, methyl ester of 9-HETE; 11, methyl ester of 11-HETE; 15, methyl ester of 15-HETE; 12, methyl ester of 12-HETE. See Figure 1 for other abbreviation.

figurations at C-5 and C-15, respectively, were determined. Thus, steric analysis of the PAP derivatives of 2-hydroxy-5-carbomethoxypentanoic acid (from the methyl ester of 5-HETE) and of 2-hydroxyheptanoic acid (from the methyl ester of 15-HETE) showed that the 5-HETE was due to 91% of the 5(*S*) enantiomer and 9% of the 5(*R*) enantiomer, whereas the 15-HETE was due to 90% of the 15(*S*) enantiomer and 10% of the 15(*R*) enantiomer.

The percentage composition of HPETE obtained following incubation of arachidonic acid with RSL-2 at pH 6.7 was: 12-HPETE, 2%; 15-HPETE, 43%; 11-HPETE, 4%; 9-HPETE, 3%; 8-HPETE, 1%; and 5-HPETE, 47% (Fig. 2A). Interestingly, the composition of HPETE obtained following incubation of arachidonic acid with RSL-2 was strongly pH-dependent. Thus, incubation at pH 9.8 afforded 15(*S*)-HPETE (88%) and 11-HPETE (9%). Formations of 5-HPETE (3%) and of 8-, 9-, and 12-HPETE, were almost completely abolished (Fig. 2B).

The methyl dihydroxyeicosatetraenoate fraction isolated by SiO_2 chromatography of the reduced and esterified product obtained following incubation of arachidonic acid with RSL-2 at pH 6.7 was subjected to radio-SP-HPLC. Two peaks of radioactivity appeared, i.e., Compound A (26.7–28.2 mL effluent; approximately 70% of the radioactivity) and Compound B (41.4–43.6 mL effluent; approximately 30% of the radioactivity). In a separate experiment, the sodium borohydride reduction was omitted. As expected, this resulted in two methyl dihydroperoxyeicosatetraenoates which eluted faster on radio-SP-HPLC, i.e., a major isomer corresponding to Compound A (18.0–19.1 mL effluent) and a minor isomer corresponding to Compound B (20.3–21.5 mL effluent).

The UV spectrum of Compound A showed λ_{max} (ethanol) = 243 nm, suggesting a 5,15-dihydroxyeicosatetraenoate having two pairs of conjugated *E,Z* double bonds (28). GC/MS analysis of the Me_3Si derivative showed a broad peak due to partial degradation on the capillary column. The mass spectrum showed prominent ions at *m/z* 479 (M – 15; loss of $\cdot CH_3$), 404 (M – 90; loss of Me_3SiOH), 394 (M – 100; rearrangement and loss of OHC-$(CH_2)_4$-CH_3), 203 [Me_3SiO^+=CH-$(CH_2)_3$-$COOCH_3$], 173 [Me_3SiO^+=CH-$(CH_2)_4$-CH_3], 129 (Me_3SiO^+=CH-CH=CH_2), and 73 (Me_3Si^+). The mass spectrum of the Me_3Si ether derivative of hydrogenated Compound A showed ions typical for a 5,15-dihydroxyeicosanoate, i.e., 487 (4%; M – 15, loss of $\cdot CH_3$), 431 [28; M – 71, loss of $\cdot(CH_2)_4$-CH_3], 402 [6; M – 100, rearrangement and loss of OHC-$(CH_2)_4$-CH_3], 401 [6; M – 101, loss of $\cdot(CH_2)_3$-$COOCH_3$], 341 [42; M – (71+90)], 311 [39; M – (101 + 90)], 203 [88, Me_3SiO^+=CH-$(CH_2)_3$-$COOCH_3$], 173 [74; Me_3SiO^+=CH-$(CH_2)_4$-CH_3], 129 (26; Me_3SiO^+=CH-CH=CH_2), and 73 (100; Me_3Si^+). Oxidative ozonolysis of the dipropionyl derivative of the methyl 5,15-dihydroxyeicosatetraenoate resulted in the formation of 2-propionoxy-5-carbomethoxypentanoic acid and 2-propionoxyheptanoic acid. Steric analysis of these compounds demonstrated that both chiral centers of the parent 5,15-dihydroxyeicosatetraenoate had the "*S*" configuration (C-5, 98.1% "*S*"; C-15, 97.9% "*S*"). This experiment also localized one double bond to the Δ^6 position and another double bond to the Δ^{13} position. On the basis of the data presented, Compound A was identified as methyl 5(*S*),15(*S*)-dihydroxy-6,8,11,13-eicosatetraenoate [methyl ester of 5(*S*),15(*S*)-diHETE].

The UV spectrum of Compound B showed λ_{max} (ethanol) 259, 268, and 279 nm, suggesting a conjugated triene structure (*cf.* Ref. 28). GC/MS analysis of the Me_3Si derivative showed a broad peak due to partial degradation. The mass spectrum showed prominent ions at *m/z* 423 [M – 71, loss of $\cdot(CH_2)_4$-CH_3], 394 [M – 100; rearrangement and loss of OHC-$(CH_2)_4$-CH_3), 243 [Me_3SiO^+=CH-CH_2-CH=CH-$(CH_2)_3$-$COOCH_3$], 173 [Me_3SiO^+=CH-$(CH_2)_4$-CH_3], and 73 (Me_3Si^+). The data mentioned suggested a 8,15-dihydroxyeicosatetraenoate, in which three of the four double bonds formed a conjugated triene. Catalytic hydrogenation afforded methyl 8,15-dihydroxyeicosanoate, as shown by the mass spectrum (Me_3Si derivative), which showed prominent ions at *m/z* 487 (5%; M – 15, loss of $\cdot CH_3$), 431 [14; M – 71, loss of $\cdot(CH_2)_4$-CH_3], 359 [13; M – 143, loss of $\cdot(CH_2)_6$-$COOCH_3$], 341 [38; M – (71 + 90)], 269 [29; M – (143 + 90)], 245 [94; Me_3SiO^+=CH-$(CH_2)_6$-$COOCH_3$], 216 [17; M – 286, rearrangement with loss of OHC-$(CH_2)_6$-CH($OSiMe_3$)-$(CH_2)_4$-CH_3], 173 [60; Me_3SiO^+=CH-$(CH_2)_4$-CH_3], and 73 (100; Me_3Si^+). Oxidative ozonolysis of the dipropionyl derivative of Compound B resulted in the formation of the propionyl derivatives of malic acid and of 2-hydroxyheptanoic acid. Steric analysis of the PAP derivatives of these compounds showed that C-8 and C-15 of the parent compound had both the "*S*" configuration (C-8, 99.0% "*S*"; C-15, 97.1% "*S*"). Furthermore, formation of the fragments mentioned, coupled with knowledge about the positions of the hydroxyl groups (at C-8 and C-15), and the presence of a conjugated tetraene structure, localized the four double bonds of Compound B to the Δ^5, Δ^9, Δ^{11}, and Δ^{13} positions. Accordingly, Compound B was identified as methyl 8(*S*),15(*S*)-dihydroxy-5,9,11,13-eicosatetraenoate [methyl ester of 8(*S*),15(*S*)-diHETE] (Fig. 3).

Products formed from arachidonic acid upon incubation with RLIL. [1-^{14}C]Arachidonic acid (100 μM) was stirred with enzyme preparation (3 mg of protein) in 0.1 M potassium phosphate buffer pH 6.7 (200 mL) at 23°C for 15 min. SiO_2 open column chromatography gave a hydroperoxide fraction accounting for approximately 90% of the radioactivity applied. The material was treated with sodium borohydride, and the esterified product was subjected to radio-SP-HPLC. A single peak due to the methyl ester of 15-HETE appeared (methyl ester of 11-HETE, less than 1%). Steric analysis of the 2-propionoxyheptanoic acid obtained following oxidative ozonolysis showed a high enantiomerical purity of the parent compound, i.e, 99.5% 15(*S*)-HETE and 0.5% 15(*R*)-HETE. Incubation of arachidonic acid with RLIL also was carried out at pH 9.8 and 5.2. In contrast to the results obtained with RSL-2, no pH-dependent change in regiospecificity could be observed. Thus, all incubations carried out in the pH range 5.2–9.8 led to the formation of 15(*S*)-HPETE with less than 3% of other HPETE isomers.

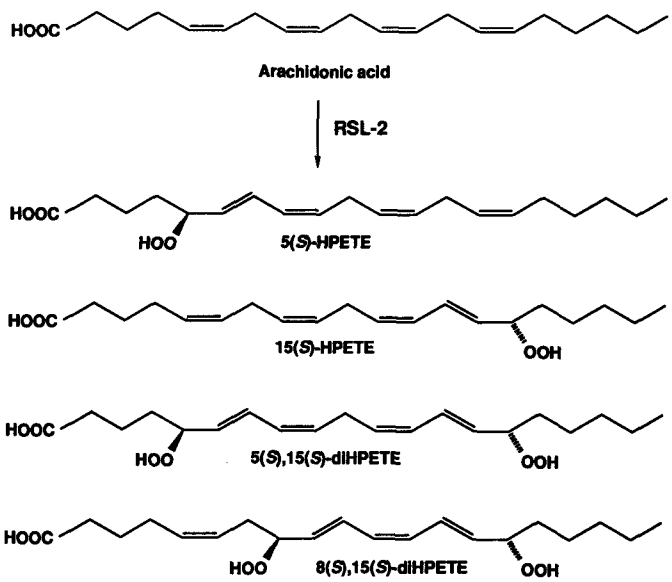


FIG. 3. Structures of mono- and dihydroperoxyeicosatetraenoates formed from arachidonic acid in the presence of RSL-2 at pH 6.7. See Figures 1 and 2 for abbreviations.

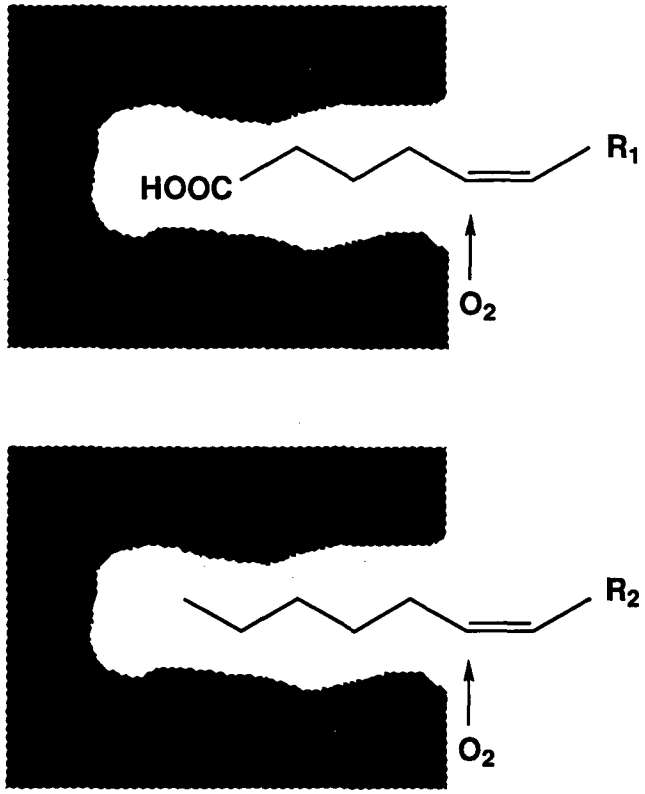


FIG. 4. Proposed head-to-tail orientations of the nonprotolyzed form of arachidonic acid at the active site of rice seed lipoxygenase-2 (RSL-2). Insertion of oxygen from a position above the plane of the carbon chain will result in the formation of 5(*S*)-HPETE when the substrate is oriented with its carboxyl end in the hydrophobic pocket (upper) and to 15(*S*)-HPETE when the orientation is reversed (lower). R_1, $(CH_2\text{-}CH{=}CH)_3\text{-}(CH_2)_4\text{-}CH_3$; R_2, $(CH_2\text{-}CH{=}CH)_3\text{-}(CH_2)_3\text{-}COOH$. See Figures 1 and 2 for abbreviations.

DISCUSSION

In agreement with previous work (18,19), RSL-2 and RLIL were found to catalyze oxygenation preferentially at the n-6 position of linoleic acid to provide 13(*S*)-HPOD as the major product. However, when arachidonic acid was used as substrate, the product profiles of the two enzymes differed. Thus, oxygenation of arachidonic acid in the presence of RLIL led almost exclusively to the formation of a single monohydroperoxide, i.e., 15(*S*)-HPETE. The enantiomerical purity of this compound was high (99.5% "*S*"). In contrast, catalysis by RSL-2 led to the formation of two major monohydroperoxides [5(*S*)-HPETE and 15(*S*)-HPETE] as well as significant amounts of two double dioxygenation products, [5(*S*),15(*S*)-diHPETE and 8(*S*),15(*S*)-diHPETE] (Fig. 3). Interestingly, the regiospecificity of RSL-2 was strongly pH-dependent. At pH 6.7, 5(*S*)-HPETE and 15(*S*)-HPETE were formed in a ratio of 52:48. Increasing the pH to 9.8 suppressed formation of 5(*S*)-HPETE and gave 15(*S*)-HPETE as the major product [ratio 5(*S*)-HPETE/15(*S*)-HPETE, 3:97]. In this context it is interesting to note that the regiochemistry of soybean lipoxygenase-1-catalyzed oxygenation of linoleic acid has been found to be pH-dependent (29), a phenomenon that is probably caused by alternate head-to-tail orientations of the substrate at the active site of the enzyme (29). It seems likely that the pH effect in arachidonic acid oxygenation observed in the present study can be explained in the same way, i.e., by the pH-dependent ionization of the carboxyl group coupled with the existence of a hydrophobic pocket in the active site of RSL-2. Thus, at an alkaline pH, the carboxyl group will exist as the charged carboxylate anion and, hence, only the ω-end of the arachidonic acid molecule can enter the hydrophobic pocket. In this situation, formation of one major HPETE [i.e., 15(*S*)-HPETE] will be predicted. On the other hand, at more acidic pH the carboxyl group will be protonated and therefore be able to enter the hydrophobic pocket. Two orientations of the substrate will thus be possible, leading to the formation of two major HPETE isomers [5(*S*)- and 15(*S*)-HPETE] (Fig. 4).

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cis/trans Isomerization of Unsaturated Fatty Acids as Possible Control Mechanism of Membrane Fluidity in *Pseudomonas putida* P8

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ABSTRACT: Exponentially growing cells of *Pseudomonas putida* had an increased ratio of saturated to unsaturated fatty acids in response to increased growth temperatures. Resting cells in which fatty acid biosynthesis was stopped reacted to a thermal increase by converting *cis*-monounsaturated fatty acids to *trans* isomers. *cis/trans* Isomerization of up to 60% of the unsaturated fatty acids was also activated by alcohols of different chain length. Their effective concentrations apparently depended on the lipophilic character of the alcohols. Also, a salt shock caused by the addition of NaCl resulted in the production of *trans* fatty acids. However, cells that were adapted to growth media of high osmolarity synthesized cyclopropane fatty acids instead of *trans* fatty acids. Activity of *cis/trans*-isomerase was dependent on the growth phase and was significantly higher during logarithmic growth than during the stationary phase. The results of this study agree with the hypothesis that the isomerization of *cis* into *trans* unsaturated fatty acids is an emergency action of cells of *P. putida* to adapt membrane fluidity to drastic changes of environmental conditions.

Lipids 31, 811–815 (1996).

The fatty acid profile of microorganisms is a stable genetic trait and can be used for chemotaxonomic purposes. Bacteria have to be cultured under standarized conditions; otherwise the fatty acid composition of lipids may vary considerably with changes in environmental conditions, growth rate, or growth phase (1,2). Apparently, these lipid modifications enable vital membrane functions to continue if physical or chemical factors influence the stability of the cell membrane. Bacteria have different mechanisms to alter lipid composition and hence the fluidity of their membranes, in response to environmental changes, such as growth temperature. Many studies show that the most important way for bacteria to regulate membrane fluidity is to increase or decrease the proportion of saturated to *cis*-monounsaturated fatty acids (1–4).

Recently, *trans* unsaturated fatty acids were detected in bacterial lipids among the commonly found *cis* isomers (5–8). These unusual membrane constituents were identified in strains of *Pseudomonas putida* which can degrade toxic pollutants, such as phenol (9,10). Cells react to the addition of different aromatic substances or ethanol with the production of *trans*-unsaturated fatty acids (11,12). The increase in the amount of fatty acids with a double bond in a *trans* configuration is accompanied by an appropriate decrease of the corresponding *cis* isomers. Biosynthesis of *trans* fatty acids is located in the cytoplasmic membrane and is apparently performed by isomerization of *cis* unsaturated fatty acids to *trans* and not by *de novo* synthesis of fatty acids (13,14).

Thermal adaptation of the phenol-degrading bacterium *P. putida* P8 is normally done by modifying the degree of fatty acid saturation (11). Here we have studied if *cis/trans* isomerization of fatty acids is also involved in the regulation of membrane fluidity. Therefore, we observed the effects on the lipid composition of factors known to influence the membrane fluidity. The isomerization of *cis* unsaturated fatty acids to *trans* is apparently a general response of *P. putida* if the environmental conditions change and the membrane composition cannot be altered by conventional mechanisms.

METHODS

Growth conditions. Pseudomonas putida P8 isolated from phenol contaminated wastewater (15) was grown at 30°C in a minimal medium that contained: 0.6 g KH_2PO_4, 1.2 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.05 g $Ca(NO_3)_2$, 0.2 g $MgSO_4$, 0.01 g $Fe(NH_4)$ citrate, 1 mL of a trace element solution (16), and 4.0 g sodium succinate per L of deionized H_2O. The pH was adjusted to 7.0. Growth of cultures was measured by optical density.

Determination of bacteriostatic and bacteriocidal alcohol concentrations. The minimal inhibitory concentration of alcohols was defined as the concentration that completely blocked cellular growth. For determination different concentrations of the alcoholic compounds were added to exponentially growing cells of *P. putida* P8 and growth was monitored by measuring the absorbance (A_{560}) of cultures. To measure bactericidal concentrations (LD_{90}), cells of the logarithmic growth phase were incubated for three hours in the presence of different concentrations of the alcohols, after which a viable count measurement was carried out. The numbers of surviving cells

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were determined, and the concentrations of alcohols needed for a tenfold reduction in cell number were calculated.

Lipid extraction and determination of fatty acid composition. Lipids were extracted with chloroform/methanol/water (1:1:1, by vol) as described by Bligh and Dyer (17). Fatty acid methyl esters were prepared by a 15-min incubation at 95°C in boron trifluoride/methanol using the method of Morrison and Smith (18). Fatty acid composition was determined using capillary gas chromatography of fatty acid methyl esters as described by Heipieper *et al.* (9). Replicate determinations indicated that the relative error (standard deviation/mean) × 100% of the values was 2–5%. The lipid fraction analyzed contained phospholipids to more than 95%.

Analysis of de novo *lipid synthesis.* Fatty acid biosynthesis was measured by studying the incorporation of labeled acetate in the lipid fraction. For this purpose 120 nCi of sodium [1-^{14}C]acetate (54 mCi/mmol; Hartmann Analytic, Braunschweig, Germany) were added to 1 mL of growing and nongrowing cells, respectively. After incubation for 2 h at 30°C, the cells were harvested and lipids were extracted as described previously. The radioactivity of the extracted lipids recovered in the chloroform layer was used as a measure of the incorporation of acetate in the fatty acids and was determined by liquid scintillation spectrometry.

Activity of cis/trans *isomerase.* Activity of *cis/trans* isomerase was measured after harvesting cultures at different times by sedimentation and resuspending cells in 50 mM potassium phosphate buffer, pH 7.0. Cells were harvested and lipids were extracted and analyzed before and after incubating with ethanol (10%, vol/vol) for 2 h at 30°C. The increase of the relative amounts of *trans* fatty acids during this activation period was defined as the *trans/cis* activity.

The *trans/cis* ratio was determined as the proportion of the sum of *trans* unsaturated to the sum of *cis* unsaturated fatty acids. The ratio of the relative amounts of saturated to unsaturated plus cyclopropane fatty acids was calculated as the degree of saturation. The experiments of this study were repeated at least three times and representative results were presented here.

RESULTS

Effect of temperature in growing cells. Membrane lipids of *P. putida* P8 cells harvested in the logarithmic growth phase contain palmitic acid (16:0) and the *cis* and the *trans* isomers of palmitoleic acid [16:1 (9)] and vaccenic acid [18:1 (11)] as main fatty acids. Additionally, myristic acid (14:0), stearic acid (18:0), and the cyclopropane fatty acids 17:0 cyc and 19:0 cyc usually occur in minor amounts (lower than 3% of total fatty acids, respectively). The lipids of these cells have a proportion of saturated to unsaturated fatty acids of about 0.6, which is increased slightly during the course of growth, and a *trans/cis* ratio below 0.1.

When the growth temperature was altered, the bacteria responded by changing the fatty acid composition and resulted in a modified degree of saturation (Fig. 1). Lipids of cells

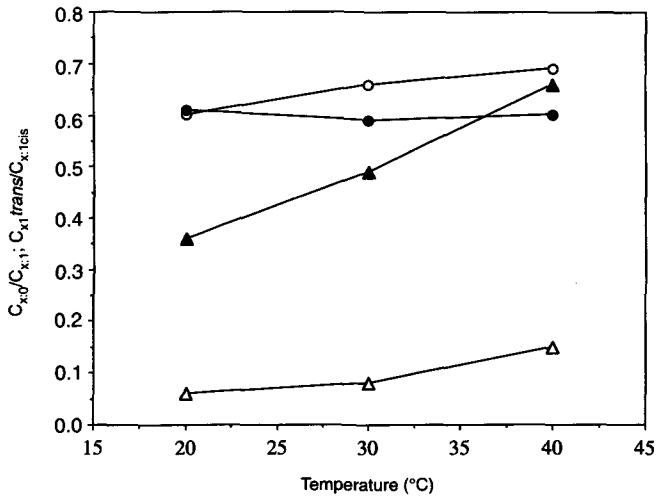


FIG. 1. Effect of temperature on the degree of saturation (circles) and the ratio of *trans* to *cis* unsaturated fatty acids (triangles) in the membrane lipids of cells of *Pseudomonas putida* P8. Exponentially growing cells were harvested by sedimentation and resuspended in growth medium (open symbols) or in 50 mM potassium phosphate buffer, pH 7 (closed symbols). After incubation for three hours at the temperature indicated, cells were harvested by sedimentation, washed with saline, and analyzed for fatty acids.

growing at the lower temperature of 20°C had a reduced degree of saturation compared with cells grown at higher temperatures. In growing cells, the ratio of *trans* to *cis* unsaturated fatty acids cells remained at a relatively low value and was only slightly increased at temperatures significantly higher than the optimal growth temperature of 30°C.

Effect of temperature in nongrowing cells. In contrast to growing cells, resting cells apparently did not use the conventional mode of thermal adaptation of lipids because they did not respond to increased temperatures with a change in the degree of saturation (Fig. 1). The cells grown at 30°C and resuspended in buffer at various temperatures converted the *cis* unsaturated fatty acids into *trans* isomers, mainly *cis* to *trans* 16:1. The increase in the *trans/cis* ratio of the unsaturated fatty acids depended on the ambient temperature and was highest at 40°C.

In the resting cells, the relative amounts of saturated fatty acids and of *cis* and *trans* unsaturated fatty acids together remained constant. Radioisotope tracer studies with sodium [^{14}C]acetate indicated that *de novo* biosynthesis of fatty acids did not occur in the nongrowing cells. Cells of *P. putida* P8 growing at 30°C in the minimal medium incorporated 13.0% of labeled acetate into lipids. Only 0.46% of the radioactivity was found in the lipid fraction after incubation with resting cells. Similiar amounts of activity were also detected in the chloroform layer during lipid extraction in control experiments without cells.

Effect of alcohols. Cis/trans conversion of up to 60% of the unsaturated fatty acids was also activated by the addition of organic chemicals, such as alcohols. These compounds were added to growing cells of *P. putida* P8. All alcohols used

were effective at different concentrations in this mode of fatty acid modification. Figure 2 shows that ethanol had to be added in a higher concentration to get the isomerization of *cis* to *trans*, while the long-chained alcohols butan-1-ol and hexan-1-ol were active in significantly smaller dosages. The effective concentrations corresponded to the length of the aliphatic moiety and thus the lipophilicity of the compounds.

The same alcoholic substance concentrations that caused maximal activation of *cis/trans* isomerization also completely inhibited cell growth (Fig. 2). At lower concentrations, the conventional mode of adaptation, i.e., change of degree of saturation was observed, and cell growth still continued. Bactericidal concentrations of alcohols used in these experiments were also measured. For ethanol (LD_{90}: 13% vol/vol) and butan-1-ol (3.0%) the lethal dose was in the range of the fatty acid modification. Either *cis/trans* isomerization was still possible in inactivated cells or conversion of unsaturated fatty acid preceeded the lethal event. In the case of hexanol (1.3%) the dose for a tenfold reduction in viable count was significantly higher than the range of concentrations inducing strong *cis/trans* isomerization of unsaturated fatty acids.

Effect of salt addition. We have studied the effect of an increase in salt concentration in the minimal medium by addition of NaCl. When cells of *P. putida* P8 were grown for several generations in the modified growth media (Fig. 3A), membrane lipids contained increased amounts of cyclopropane fatty acids, especially 17:0 cyc, while the content of the *cis* unsaturated fatty acids was diminished. The relative amounts of saturated and of *trans* fatty acids were not influenced.

The fatty acid composition of cellular lipids was modified in a completely different way if cells could not adapt to the increased salinity of the medium (Fig. 3B). In these experiments cells growing in minimal medium without salt were shocked by the addition of NaCl. These cells also responded to the increased salt concentration with a decrease of *cis* unsaturated fatty acids. In this case, a production of *trans* and not of cyclopropane fatty acids followed the change in medium composition.

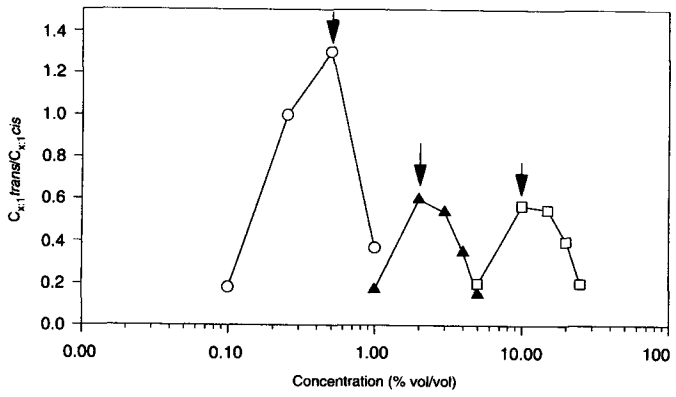


FIG. 2. Effect of ethanol (□), butanol (▲), and hexanol (○) on the ratio of *trans* to *cis* unsaturated fatty acids in the membrane lipids of cells of *Pseudomonas putida* P8. Alcohols were added to growing cells in the late log phase. After incubation for 3 h at 30°C cells were harvested, lipids were extracted and analyzed for fatty acids. Minimal inhibitory concentrations of the alcohols are indicated by an arrow above the plot of fatty acids.

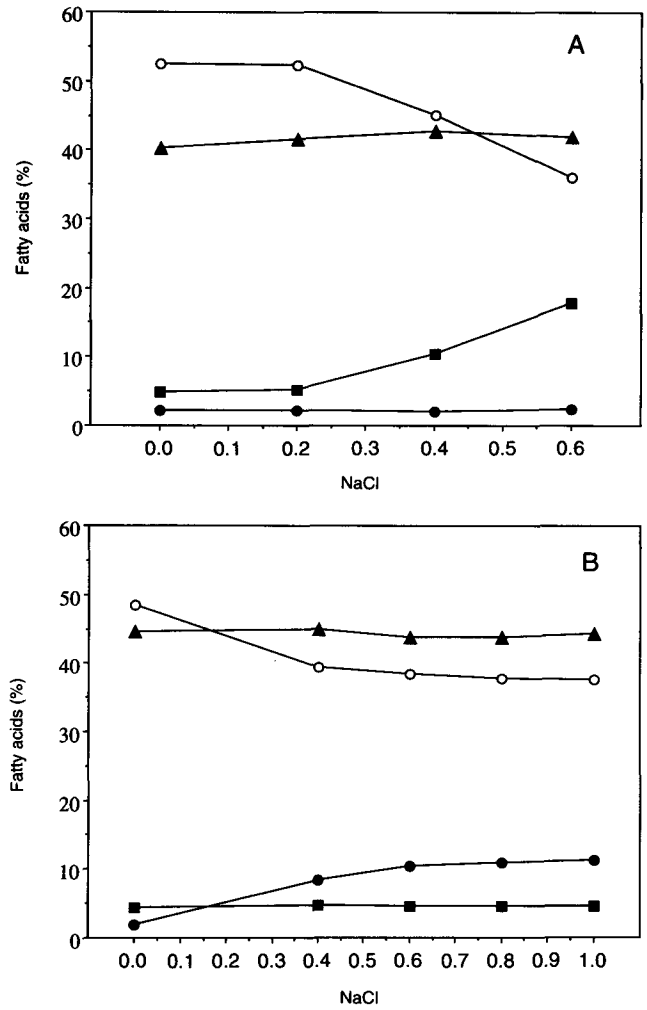


FIG. 3. Effect of salinity on fatty acid composition of salt-adapted and nonadapted cells of *Pseudomonas putida* P8: *trans* unsaturated (●), *cis* unsaturated (○), cyclopropane (■), and saturated fatty acids (▲). A, Salt-adapted cells were grown as overnight and main cultures in growth media with different salt concentrations. Cultures were harvested in the late-log phase and the bacterial lipids were analyzed. B, Cells were grown in a medium without NaCl and shocked by addition of concentrated salt solutions. Cells were sedimented 2 h later and analyzed for fatty acids.

Effect of growth phase. Pseudomonas putida cells at the stationary phase of cell growth were harvested and their lipids analyzed. They had a fatty acid composition significantly different from that of cells in the log growth phase (Fig. 4). The lower proportion of *cis* unsaturated fatty acids was mainly caused by an increase in the amounts of cyclopropane fatty acids. Simultaneously, the cells had a smaller content of *trans* fatty acids than the exponentially growing cells.

A conclusion about the activity of *cis/trans*-isomerase can be made by comparing the *trans* content of cells activated by ethanol with that of untreated cells. Figure 5 shows that this activity is dependent on the growth phase of the cells. Loga-

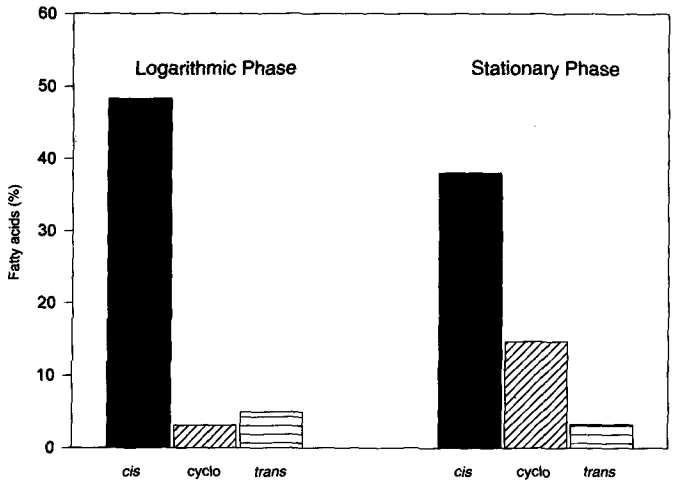


FIG. 4. Relative contents of fatty acids in cells of *Pseudomonas putida* P8 harvested in the mid-log phase (left three bars) or three hours after entry in the stationary phase (right three bars). Lipids were extracted and analyzed for *cis* unsaturated, cyclopropane (cyclo), and *trans* unsaturated fatty acids.

rithmically growing cells incubated with ethanol had a high activity of fatty acid conversion; a maximum was measured in the mid-log phase. Cells harvested in the late log phase showed a significantly decreased activity. In the stationary phase activity of *cis/trans*-isomerase dropped to a low level. Apparently, the isomerization of *cis* to *trans* fatty acids is not only influenced by environmental conditions, but also by intrinsic factors.

DISCUSSION

Fatty acid composition of bacterial lipids is affected by temperature (2,4,6–8,19), the presence of organic compounds such as aromatics or alcohols (20–23), or the osmolarity of the growth medium (24–26). Cells of *P. putida* P8 also respond to these membrane-active stimuli with commonly found membrane modifications, such as an increase in the degree of saturation of fatty acids or in the proportion of cyclopropane fatty acids. This study shows that the environmental factors, if applied under growth-inhibiting conditions, induce conversion of *cis* into *trans* unsaturated fatty acids in this bacterial strain. The isomerization of a large portion of *cis* into *trans* unsaturated fatty acids markedly decreases the membrane fluidity, as shown by different studies (8,10,27). Obviously, the reason for this is that the steric behavior of *trans* unsaturated fatty acids in membranes is similiar to that of saturated acyl chains. Both types of fatty acids possess a long extended conformation and a small molar volume as membrane constituents. In contrast to these acyl chains, *cis* unsaturated fatty acids have a rigid bend with an angle of 30°, and interrupt the highly ordered package of the acyl chains, leading to lower phase transition temperatures of the membrane.

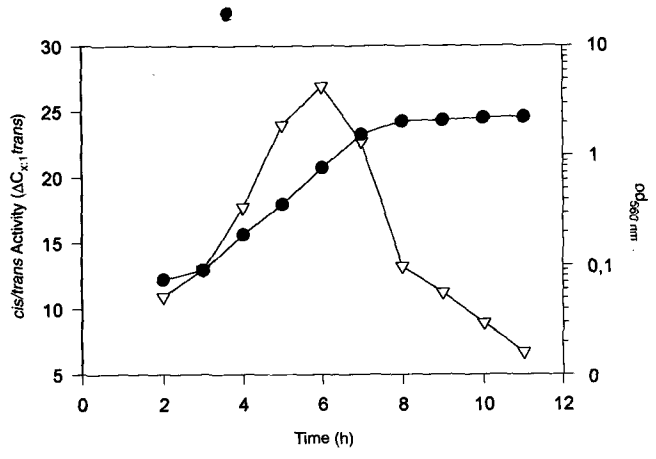


FIG. 5. Activity of *cis/trans* isomerase (▽) during growth (●) of *Pseudomonas putida* P8.

The regulation of membrane fluidity by modification of the ratio of saturated to monounsaturated fatty acids is probably restricted for growing cells in bacteria using the anaerobic way of fatty acid biosynthesis like *P. putida* and *Escherichia coli* (28). These bacteria, which lack membrane-bound desaturases, completely synthesize the saturated and unsaturated acyl chains of lipids in the cytoplasma. If cell growth ceases, lipid and fatty acid biosynthesis are immediately stopped and the degree of saturation cannot significantly be manipulated by the cells (29,30). Those bacteria that have the capacity of converting the double bond configuration possess a mechanism to change membrane fluidity, that is independent of growth. The mechanism is used whenever environmental conditions change so drastically that growth cannot continue.

An increase in the portions of cyclopropane fatty acids in cellular lipids is also frequently assessed when batch cultures enter the stationary phase of growth (1,28,31). The effect is apparently caused by the stringent response (32). Contrary to the activity of cyclopropane fatty acid synthetase, the activity of the *cis/trans* isomerase decreased significantly in *P. putida* P8 cells of the stationary phase. This observation is not in agreement with that of Guckert *et al.* (31). After long-term starvation of cells of *Vibrio cholerae* these authors measured increased amounts of *trans* unsaturated fatty acids. They explained this by a greater metabolic stability of the *trans* fatty acids than *cis* isomers in starving cells. However, cells of *Shewanella putrefaciens* and *Vibrio* sp. also showed a lower content of *trans* unsaturated fatty acids when their lipids were analyzed in the stationary growth phase (7,33). Therefore, there is more evidence that *cis/trans* isomerization is an emergency system of growing cells induced by threatening environmental conditions rather than a system that protects microbial lipids from degradation during nutritional deprivation.

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Alterations in Cell Cholesterol Content Modulate Ca^{2+}-Induced Tight Junction Assembly by MDCK Cells

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ABSTRACT: Transepithelial electrical resistance (TER), a measure of tight junction (TJ) barrier function, develops more rapidly and reaches higher values after preincubation of MDCK cells for 24 h with 2 μM Lovastatin (lova), an inhibitor of 3-hydroxy-3-methylglutaryl–CoA reductase. While this effect was attributed to a 30% fall in cholesterol (CH), possible effects of lova on the supply of prenyl group precursors could not be excluded. In the current study, strategies were devised to examine effects on TER of agents that simultaneously lower CH and increase the flux of intermediates through the CH biosynthetic pathway. Zaragozic acid, 20 μM, an inhibitor of squalene synthase known to increase the synthesis of isoprenoids and levels of prenylated proteins, lowered cell CH by 30% after 24 h, while accelerating development of TER in the same manner as lova. TER was also enhanced, despite a 23% increase in the rate of [^{3}H]acetate incorporation into CH, when total CH was reduced by 45% during a 2-h incubation with 2 mM methyl β-cyclodextrin (MBCD), an agent that stimulates CH efflux from cells. The fact that the rate of TER development was diminished when cell CH content was elevated by incubation with a complex of CH and MBCD is further evidence that this sterol modulates development of the epithelial barrier. Cell associated CH derived from the complex was similar to endogenous CH with respect to its accessibility to cholesterol oxidase. Lova's effect on TER was diminished when 5 μg/mL of CH was added to the medium during the last 11 h of incubation with lova.
Lipids 31, 817–828 (1996).

Movement of solutes and water from one compartment to another across an epithelial monolayer occurs *via* transcellular and/or paracellular pathways. Flux through the latter is determined by tight junctions (TJ), the most apical component of the epithelial junctional complex (1). Several proteins, including occludin, the first to have structural features and a subcellular distribution expected of a TJ protein (2), have been implicated in TJ assembly and function. The role of plasma membrane lipids in TJ biology is not as well understood. Early freeze fracture electron microscopy studies revealed striking similarities between TJ particles and structures formed after addition of Ca^{2+} to defined phospholipid mixtures (3–5). Based on these observations it was proposed that the TJ particles themselves might represent tubular, inverted, lipid micelles formed by fusion of the outer lipid bilayer of adjacent cells at points where they make contact. Although direct experimental support for this model is lacking, under certain conditions a lipid-soluble probe can be made to diffuse directly from the apical surface of one cell to that of another in a monolayer with established TJ (6,7).

Whether they constitute a structural element of the TJ or not, plasma membrane lipids are a diverse mixture of molecules which, if altered, can affect the activity of a variety of plasma membrane proteins (8,9), as well as more macroscopic events, such as the fusion of membranes (10). Directly or indirectly, therefore, plasma membrane lipids may affect TJ assembly and function. Attempts have been made to relate alterations in cell phospholipid composition to changes in transepithelial electrical resistance (TER), a commonly used measure of TJ function (11). Modifications in phospholipid fatty acyl group composition, for example, caused by the addition of exogenous fatty acids to the culture medium, altered the kinetics of Ca^{2+}-induced TJ assembly (12). Changes in phospholipid head group composition, on the other hand, had little effect on TJ (13). The compositional alterations produced in these studies were not confined to the plasma membrane, however, but were probably more global. In a recent study changes more localized to the cell surface lipid environment were induced by taking advantage of the observation that up to 90% of the cell's cholesterol (CH) is located in the plasma membrane (14,15). By incubating cells with Lovastatin (lova), an inhibitor of 3-hydroxy-3-methylglutaryl (HMG)–CoA reductase, most of any significant decline in total cell CH should occur in the plasma membrane pool. Decreasing CH by 30% in this fashion halved the time for initiating the development of TER and resulted in higher peak values when TJ were induced to form by addition of Ca^{2+} (16).

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Abbreviations: BCS, bovine calf serum; CH, cholesterol; CH-MBCD, cholesterol-methyl β-cyclodextrin; DMEM, Dulbecco's modified Eagle's medium; EBSS, Earle's balanced salt solution; HMG, 3-hydroxy-3-methylglutaryl; lova, Lovastatin; MBCD, methyl β-cyclodextrin; PBS, phosphate-buffered saline; P/S, penicillin/streptomycin; S.A., specific activity; TCA, trichloroacetic acid; TER, transepithelial electrical resistance; TJ, tight junction; TLC, thin-layer chromatography.

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Because some of the intermediates derived from mevalonate are common precursors for biosynthesis of both CH and prenyl groups, the effects of lova on TER may not have been related solely to changes in CH content. In fact, among the proteins which undergo prenylation, rab 13, a small GTP binding protein, was recently shown to colocalize with the TJ associated protein zonula occludens-1 in polarized epithelial cells with formed TJ (17). Several observations, however, suggest that the fall in cell CH, rather than a decrease in the availability of precursors for prenyl group synthesis, is responsible for lova's effect on TER. The concentration of lova that produced accelerated Ca^{2+}-induced TJ assembly, for example, was considerably less (2 μM) than that commonly used to inhibit prenylation of proteins in a variety of cells (20–30 μM) (18–20). Furthermore, lova's effect on TER was overcome only at high concentrations of mevalonate (5 mM) that restored CH levels to control values, and not at lower levels (0.5 mM), which do not increase cell CH but do support prenylation of proteins (16,18,20). Although these data are consistent with the conclusion that TJ assembly is sensitive to the CH content of the plasma membrane, the possibility remains that lova's effect on TJ assembly might, at least in part, result from a decrease in the availability of intermediates required for protein prenylation.

In the present study strategies were employed to examine further the relationship between cell CH content and the development of TER. In one, zaragozic acid, a competitive inhibitor of squalene synthase known to increase the level of precursors utilized for isoprenoid synthesis (21,22), was substituted for lova and its effect on TER determined. As an alternative approach, instead of using CH synthesis inhibitors, a cholesterol binding agent, methyl β-cylcodextrin (MBCD), or a complex of CH and MBCD (CH-MBCD) was added to the medium to lower or raise, respectively, cell CH content. The data presented show that the CH taken up from the CH-MBCD complex is similar to endogenous CH with respect to its preferential accumulation in the plasma membrane. At low concentrations, exogenous CH (as CH-MBCD complex) overcomes, to a large extent, lova's effect on cell CH content and the development of TER.

METHODS AND MATERIALS

Reagents. Lova and zaragozic acid A were gifts from Merck Sharp and Dohme Research Labs (Rahway, NJ). Dulbecco's modified Eagle medium (DMEM), Ham's F-12 nutrient mixture, Earle's balanced salt solution (EBSS), and individual components to make low Ca^{2+} minimal essential medium (low Ca^{2+} medium) were from Gibco (Grand Island, NY). Newborn bovine calf serum (BCS) was from Hyclone Laboratories (Logan, UT), and trypsin was from Worthington Diagnostic Systems (Freehold, NJ). Trizma base, EDTA, sodium taurocholate, carbowax 6000, *p*-hydroxyphenylacetic acid, horseradish peroxidase (type VI; EC 1.11.1.7), *o*-dianisidine, CH-MBCD, MBCD, CH, and CH oxidase (*Streptomyces* species; 25 activity units per mg; EC 1.1.3.6) were obtained from Sigma Chemical Co. (St. Louis, MO). Radioactive reagents [^{3}H]acetic acid, sodium salt [specific activity (S.A.) 135.4 mCi/mmol], [4-^{14}C]CH (S.A. 54.0 mCi/mmol), and [methyl-^{3}H]thymidine (S.A. 84.2 Ci/mmol), were purchased from New England Nuclear Corp. (Boston, MA). Thin-layer chromatography (TLC) plates (Redi/Plate, Silica Gel G) and all organic solvents were from Fisher Scientific (Pittsburgh, PA). Two types of tissue culture inserts were used: Millicell-HA from Millipore (Bedford, MA) and Nunc 25-mm from Nunc Inc. (Naperville, IL). All chemical reagents were of analytical grade.

Cell culture. MDCK cells (CCL-34), obtained from the American Type Tissue Culture Collection (Rockville, MD), were used at passage levels 56–72. They were maintained in antibiotic-free DMEM/Ham's F-12 supplemented with 10% BCS and subcultured weekly at a split ratio of 1:6 using 0.05% trypsin in 0.02% EDTA in Ca^{2+}, Mg^{2+}-free EBSS, pH 7.2. Unless otherwise noted, cultures were kept at 37°C in a humidified atmosphere of 95% air and 5% CO_2; culture medium was changed at 2–3-d intervals. Stock cultures were routinely monitored for the presence of mycoplasma. Culture medium for experiments was supplemented with 500 U/mL penicillin and 0.5 mg/mL streptomycin (P/S).

Inhibitor stock solutions. Stock solutions of 6 mM lova (molecular weight 404.5) and 20 mM zaragozic acid (molecular weight 756) were freshly prepared in ethanol just prior to use.

"Ca^{2+} Switch" protocol. MDCK cells were plated at 1.5 times the number at confluence on inserts in DMEM/Ham's F-12, 10% BCS. Three hours later, floating cells were removed by rinsing with Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS), and adherent cells cultured in low Ca^{2+} medium, 1% BCS, P/S for 48 h. Assembly of TJ was then initiated by changing to Ca^{2+}-sufficient medium (low Ca^{2+} medium, 1% BCS, P/S supplemented with 1.8 mM $CaCl_2$) (23).

TER measurements. For repeated, sequential TER measurements on individual monolayers grown on 0.6-cm^2 Millicell-HA inserts, a Millicell-ERS volt-ohmmeter (World Precision Instruments, New Haven, CT) was utilized under temperature-controlled conditions at 37°C with electrodes reproducibly placed. Precautions were taken to maintain sterility during TER measurements. In our hands, the MDCK cells are well anchored to the Millicell-HA membrane and sample-to-sample variability in TER values is minimized (16,24).

Thymidine uptake studies. One hour before and at various times after the addition of Ca^{2+}, lova-treated and control cells were radiolabeled for 1 h with 1.0 μCi/mL [^{3}H]thymidine. Monolayers were washed to remove residual [^{3}H]thymidine and incubation in nonradioactive medium continued until the assay was terminated on day four. To collect the radioactive DNA, monolayers were rinsed six times with ice-cold PBS and lysed with 1 mL of 0.1% SDS for 15 min at 37°C. Lysates were collected and aliquoted as follows—200 μL for protein determination and 800 μL for trichloroacetic acid (TCA) precipitation. Ten mL of ice cold 5% TCA was added to the lysate for 10 min. The TCA precipitates were collected on AA 0.8-

μm filters (Millipore) using a manifold (Millipore), washed twice with cold 5% TCA, air-dried, and transferred to scintillation vials. Three mL of Ultima Gold (Packard, Middletown, CT) was added and the samples were counted in a 2200CA Tri Carb Liquid Scintillation Analyzer (Packard).

Metabolic labeling of endogenous CH. Cells were cultured on Nunc 25-mm tissue culture inserts in DMEM/Ham's F-12, 10% BCS; after 3 h the medium was replaced with low Ca^{2+} medium, 1% BCS, P/S supplemented with 1.0 μCi/mL [^{3}H]acetate or 0.025 μCi/mL [^{3}H]acetate for double labeling experiments. The Nunc inserts were chosen for two reasons—first, they are transparent and therefore allow the monolayer to be monitored by phase microscopy and, second, the substratum, which is resistant to organic solvents, can be readily removed and monolayer lipids extracted directly. Although there was more variation in TER measurements from insert to insert, CH measurements, as well as the overall time-course for Ca^{2+}-induced TJ formation and final steady-state TER values, were similar to those made on Millicell-HA inserts.

Preparation of CH-MBCD in cell culture medium. For cell culture applications an aqueous solution of CH-MBCD was prepared as recommended by Sigma Chemical Co. (25). Briefly, an aqueous stock solution of CH-MBCD equivalent to 5 mg/mL CH (0.109 g complex/mL) was filtered in a sterile manner and stored at 4°C. An aliquot of the stock solution was diluted with cell culture medium to obtain the desired final concentration of CH. The final molar ratio of CH to MBCD was 1:6.2, and the concentration of MBCD used during supplementation with cholesterol varied from 0.02–0.09 mM.

Preparation of radioactive CH-MBCD. A mixture containing 15 mg CH in ethanol and 5 μCi of ethanolic [^{14}C]CH was dried under a stream of N_2 and then heated under vacuum for 20 min at 50°C to remove all traces of solvent. The radioactive CH was moistened with a few drops of 45% MBCD in 0.9% NaCl and mixed with a glass rod until a paste-like slurry was formed. The total volume of 45% MBCD added to the slurry was adjusted to 1.0 mL and the mixture stirred overnight at room temperature. The next day it was transferred to an Eppendorf tube and microfuged for 5 min; the supernatant containing the water-soluble radioactive complex was stored at 4°C until needed (26–28). Working solutions were prepared by diluting the stock solution to a desired final concentration in cell culture medium. The resulting specific activity of the prepared inclusion complex was 561 dpm/μg CH and the molar ratio of CH to MBCD was 1:8.6.

Oxidation of CH in intact MDCK cell monolayers. Monolayers were fixed *in situ* with 1% glutaraldehyde in mannitol/Tris/$CaCl_2$ (300:12:0.5 molar ratio), pH 7.4 (mannitol buffer), for 15 min at 4°C. After fixation, they were rinsed three times with mannitol buffer, and 5 U/mL of CH oxidase at 37°C was added to the apical, the basolateral, or to both sides of the monolayers. Based on preliminary experiments which showed that oxidation of CH reached a plateau between 4 and 6 h of incubation at 37°C, samples for analysis were harvested for up to 6 h. At each time point the enzyme reaction was terminated by washing the monolayers twice with ice-cold mannitol buffer (29), removing the substratum and adherent monolayer from the insert and transferring it to a glass conical centrifuge tube containing the solvent mixture for lipid extraction.

Lipid extraction. Monolayers attached to the Nunc support were extracted three times with isopropanol/hexane (2:3, vol/vol). Extracts were dried with a stream of N_2 and the lipids redissolved in 1.0 mL of chloroform/methanol (2:1, vol/vol). A 20-μL aliquot of each sample was transferred to a scintillation vial to obtain total lipid radioactivity. CH, CH ester, and cholestenone standards were added to the samples to facilitate their visualization on the chromatography plate. The samples were dried under N_2 and dissolved in 60 μL of chloroform/methanol for transfer to a TLC plate (29).

TLC. Samples were applied to silica gel TLC plates which had been activated by heating for 45 min at 104°C. Lipids were separated using hexane/ether/acetic acid (80:20:1, by vol) as the developing solvent. Lipid spots were detected by lightly spraying the TLC plate with 0.1% aqueous 8-anilino-1-naphthalene-sulfonic acid while viewing under ultraviolet light at 366 nm. Spots corresponding to CH, CH ester, and cholestenone were scraped and transferred to scintillation vials. Ultima Gold (2 mL) was added and the samples counted. The percent oxidation was calculated as: dpm cholestenone/(dpm cholestenone + dpm CH) × 100.

CH determination. Cell monolayers were lysed and CH content determined enzymatically on a measured aliquot as previously described (16,30).

Protein determination. Protein content of cell lysates was assayed using the micro bicinchoninic acid method (Pierce, Rockford, IL) according to the manufacturer's instructions.

CH oxidase assay. Determination of CH oxidase activity in culture media was adapted from a method described by Sigma Quality Control, which was based on patent to Eastman Kodak Company (Rochester, NY) (31). Briefly, in a 1.5-mL reaction mixture with 500 μL of sample, the final concentrations of reagents are 0.009% *o*-dianisidine, 0.017% (wt/vol) CH, 0.33% (vol/vol) Triton X-100, and 10 units of horseradish peroxidase. The increase in absorbance at 500 nm was recorded for 5 min where ΔA_{500}/min was determined at the maximum linear rate.

RESULTS

Reducing cell CH using inhibitors of its synthesis: Lova. Development of TER following the addition of Ca^{2+} to Ca^{2+}-deficient monolayers of MDCK cells previously incubated for 24 h with 2 μM lova, an inhibitor of HMG–CoA reductase, occurs more rapidly than in control cells (Fig. 1A). Three hours after Ca^{2+} addition, TER rose to values approximately three times greater than control monolayers. In both experimental and control cultures, peak TER was achieved 8–10 h after addition of Ca^{2+}; however, the peak TER values attained by lova-treated monolayers were 1.5-times greater than controls. TER approached control values (90–100 $\Omega \cdot cm^2$) by 48 h. Parallel sets of control and lova-treated monolayers har-

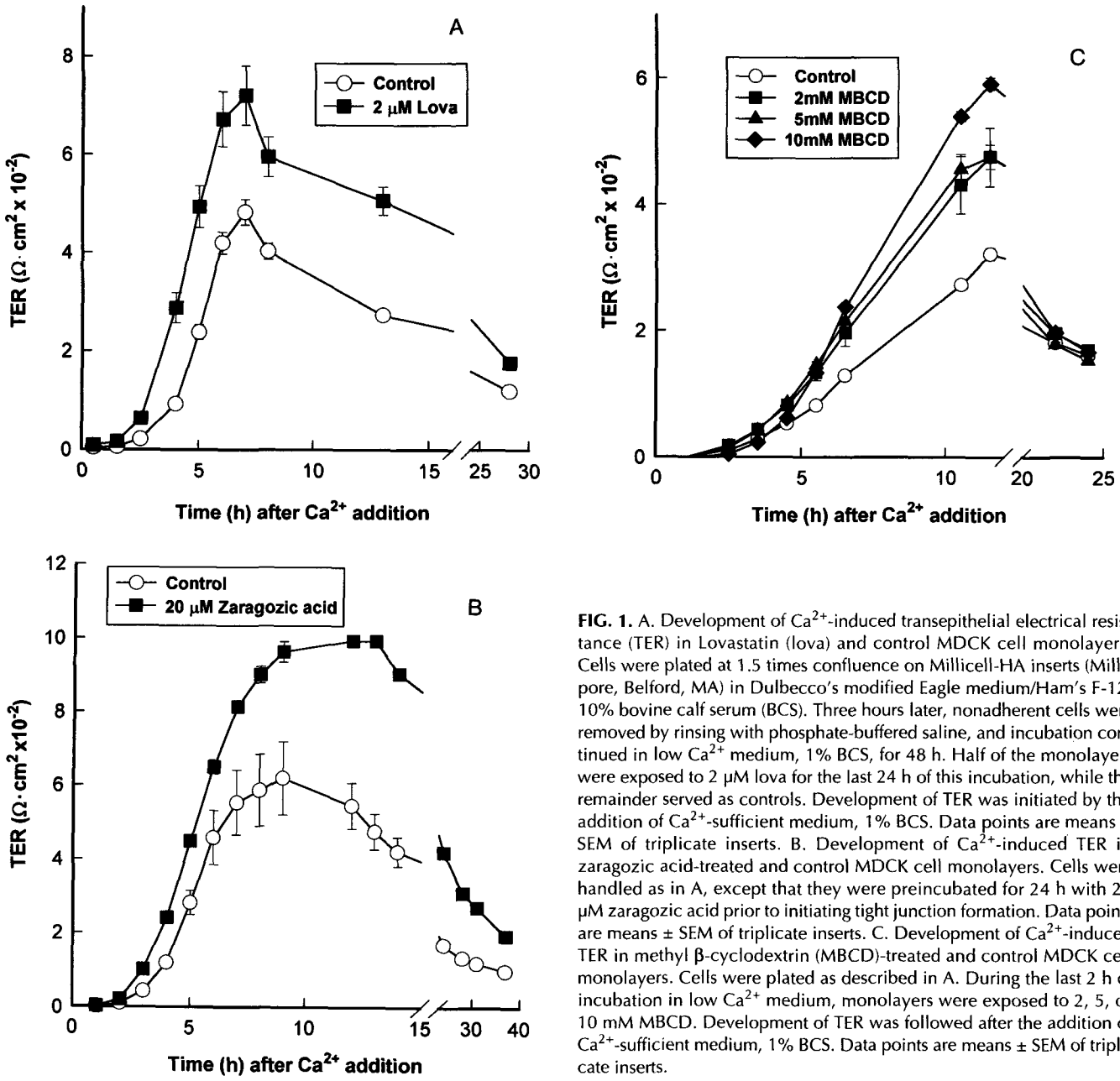


FIG. 1. A. Development of Ca^{2+}-induced transepithelial electrical resistance (TER) in Lovastatin (lova) and control MDCK cell monolayers. Cells were plated at 1.5 times confluence on Millicell-HA inserts (Millipore, Belford, MA) in Dulbecco's modified Eagle medium/Ham's F-12, 10% bovine calf serum (BCS). Three hours later, nonadherent cells were removed by rinsing with phosphate-buffered saline, and incubation continued in low Ca^{2+} medium, 1% BCS, for 48 h. Half of the monolayers were exposed to 2 μM lova for the last 24 h of this incubation, while the remainder served as controls. Development of TER was initiated by the addition of Ca^{2+}-sufficient medium, 1% BCS. Data points are means ± SEM of triplicate inserts. B. Development of Ca^{2+}-induced TER in zaragozic acid-treated and control MDCK cell monolayers. Cells were handled as in A, except that they were preincubated for 24 h with 20 μM zaragozic acid prior to initiating tight junction formation. Data points are means ± SEM of triplicate inserts. C. Development of Ca^{2+}-induced TER in methyl β-cyclodextrin (MBCD)-treated and control MDCK cell monolayers. Cells were plated as described in A. During the last 2 h of incubation in low Ca^{2+} medium, monolayers were exposed to 2, 5, or 10 mM MBCD. Development of TER was followed after the addition of Ca^{2+}-sufficient medium, 1% BCS. Data points are means ± SEM of triplicate inserts.

vested at T_0, when Ca^{2+} was added, were analyzed for their CH and protein content. CH levels in the lova-treated cells were reduced by approximately 30%, and the protein content was unaffected (Table 1).

Zaragozic acid. If lova's effect on TER is more closely related to a fall in CH than to a decreased supply of prenyl group precursors, then zaragozic acid, which elevates the level of those precursors but depresses CH content (21,22,32), should also increase TER. The data in Figure 1B and Table 1 show that zaragozic acid induced a 32% fall in CH and affected the development of TER by monolayers in a manner similar to that observed with lova.

MBCD reduces cell CH by stimulating its efflux. To eliminate nonspecific effects associated with long-term exposure of cells to inhibitors of CH synthesis, another agent, MBCD, was employed to decrease cell CH. Using this cell impermeant CH binding agent at 2, 5, or 10 mM to stimulate CH efflux, CH content was diminished by 46–78% within 2 h (Table 1). The effect of MBCD on TER was virtually the same as that obtained when lova or zaragozic acid was employed (Fig. 1C). After 11 h in Ca^{2+}-sufficient medium, peak TER values attained by monolayers treated with MBCD were 1.5–1.8 times greater than controls. Lowering cell CH by stimulating efflux would be expected to stimulate production of mevalonate and endogenous cholesterol (21,22). Indeed, although the rate of [^{3}H]acetate incorporation into CH by cells incubated for 24 h with lova decreased below control values, that of cells treated with MBCD for 2 h actually increased (Fig. 2).

Raising cell CH content depresses the ability of MDCK cell monolayers to develop TER. The uptake of [^{14}C]CH (5

TABLE 1
Protein and Cholesterol Content of MDCK Cell Monolayers Following the Administration of Agents That Reduce Cell Cholesterol (CH) Content[a]

Agent	Incubation time (h)	Protein (mg/insert)	CH (μg/insert)	μg CH/mg protein
0	24	0.52 ± 0.02	10.09 ± 0.03	19.53 ± 0.74
2 μM Lovastatin	24	0.52 ± 0.01	7.86 ± 0.24	15.08 ± 0.25
0	24	0.44 ± 0.02	9.95 ± 0.32	22.61 ± 0.95
20 μM Zaragozic acid	24	0.46 ± 0.01	7.16 ± 0.06	15.56 ± 0.29
0	2	0.56 ± 0.01	10.60 ± 0.75	19.08 ± 1.12
2 mM MBCD	2	0.58 ± 0.01	5.91 ± 0.24	10.18 ± 0.39
5 mM MBCD	2	0.55 ± 0.01	3.04 ± 0.35	5.53 ± 0.58
10 mM MBCD	2	0.55 ± 0.01	2.36 ± 0.17	4.27 ± 0.32

[a]Data are expressed as the mean ± SEM of triplicate inserts. MBCD, methyl β-cyclodextrin.

μg/mL) from a complex of [^{14}CH]-MBCD (final [MBCD] = 0.02 mM) by MDCK cells was half maximum at 5 h and peaked between 12 and 24 h (Fig. 3). The effect of increasing cell CH content on development of TER was examined by exposing monolayers to three different concentrations of CH (as the CH-MBCD complex) for either the last 11 or 24 h of a 48-h period that preceded the addition of Ca^{2+} to initiate TJ assembly. Cells from one set of inserts were subsequently analyzed for CH and protein content, whereas those on another received 1.8 mM Ca^{2+}; TER was monitored until values approached steady state. Within each of these two experiments the increase in CH content, after exposure to increasing concentrations of exogenous CH, semiquantitatively correlated with a lowering in TER values. A greater increase in CH content is associated with a larger fall in TER (Fig. 4). Interestingly, after 11 h of incubation, the data suggest that cell CH had reached a maximum of *ca.* 30 μg/mg of protein (Table 2). Evidence that the cells may have become saturated with free

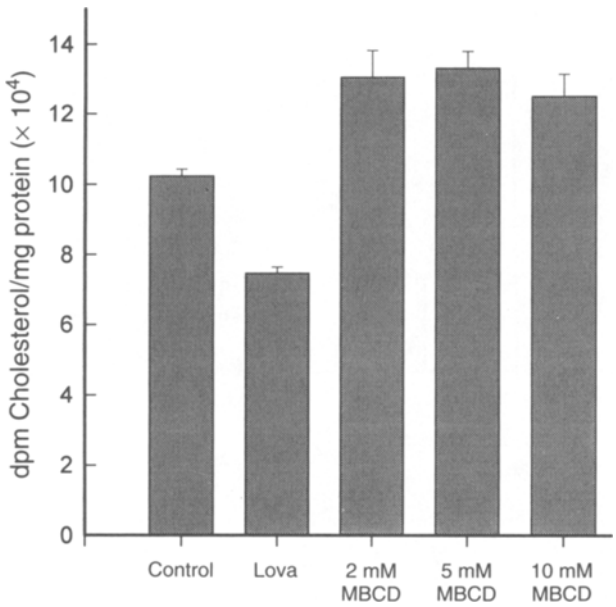


FIG. 2. [^{3}H]Acetate incorporation into cholesterol (CH) of control, lova-, and MBCD-treated cells. Cells were plated at confluence in Dulbecco's modified Eagle's medium/Ham's F-12, 10% BCS. Three hours later, they were transferred to low Ca^{2+} medium, 1% BCS. One day after plating one set of inserts received 2 μM lova while the remainder had their medium changed. On the third day after plating, other sets of monolayers were exposed either to 2, 5, or 10 mM MBCD for 2 h. At the end of that time all sets were pulse-labeled for 15 min with 1.0 μCi/mL [^{3}H]acetate. Lipids were extracted and [^{3}H]CH was isolated by thin-layer chromatography and its radioactivity assayed. Data are means ± SEM of triplicate inserts. See Figure 1 for abbreviations.

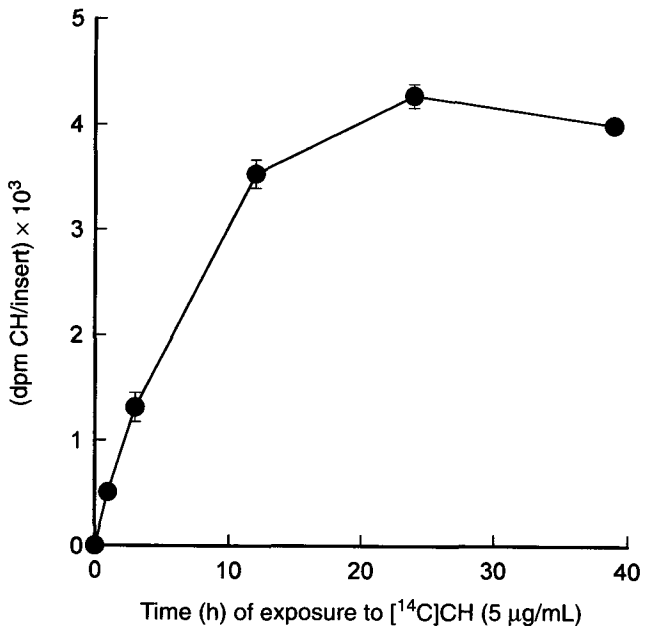


FIG. 3. Rate of [^{14}C]CH uptake by MDCK cells supplemented with [^{14}C]CH-MBCD. Cells were plated on inserts in Dulbecco's modified Eagle's medium/Ham's F-12, 10% BCS. Three hours later, nonadherent cells and medium were removed and low Ca^{2+} medium, 1% BCS, added. Three days after plating the cells were incubated with 5 μg/mL [^{14}C]CH. Lipids were extracted from cells harvested 1, 3, 12, 24, and 39 h later after extensive rinsing with phosphate-buffered saline to remove excess [^{14}C]CH-MBCD. CH was isolated by thin-layer chromatography and its radioactivity assayed as described in the Methods and Materials section. Data points are means ± SEM from six inserts. See Figures 1 and 2 for abbreviations.

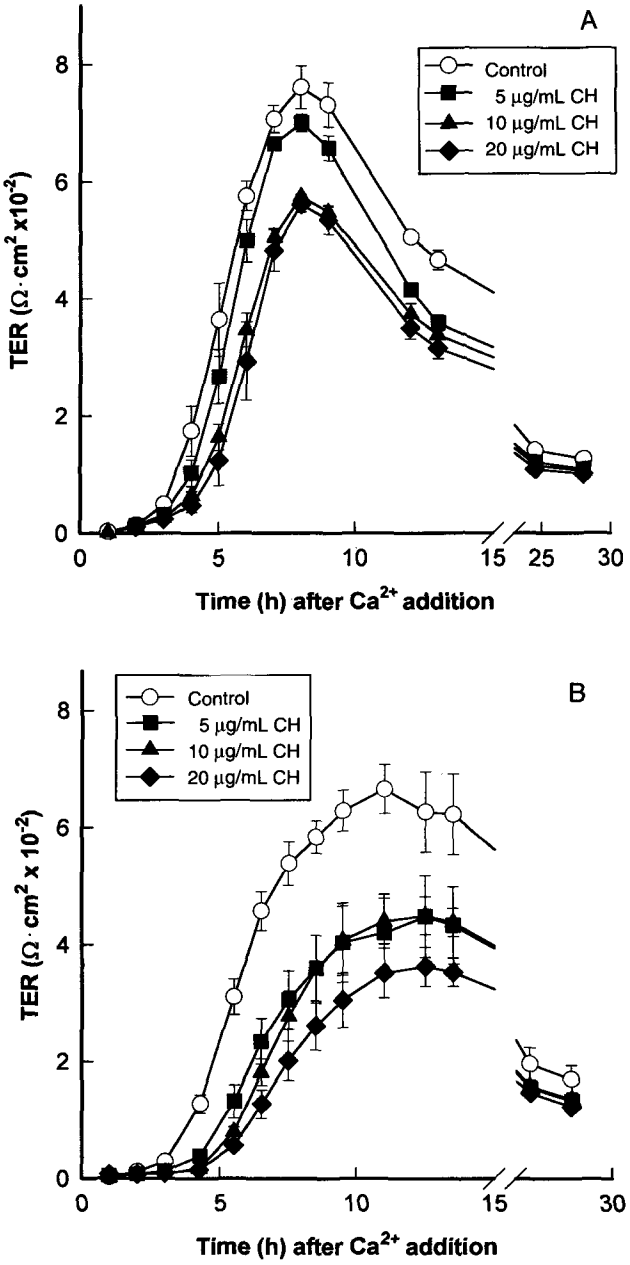


FIG. 4. Effect of exogenous cholesterol on the development of TER. Cells were plated on Millicell-HA inserts as described in the legend to Figure 1. A: Eleven hours before the addition of Ca^{2+}, medium was changed to one containing 0, 5, 10, or 20 µg/mL CH as CH-MBCD complex. B: Cells were treated as in A, except that monolayers were exposed to CH 24 h before the addition of Ca^{2+}. After addition of Ca^{2+}-sufficient medium, to all monolayers, measurements of TER were made. Data points are means ± SEM of triplicate inserts. See Figures 1 and 2 for abbreviations.

CH at this point is suggested by the fact that the fraction of exogenous CH in the form of CH ester was elevated *ca.* 3.5-fold when the exogenous CH was raised from 5 to 10 µg/mL (Fig. 5, hatched bars). Exposure of cells to CH-MBCD for 24 h resulted in greater suppression of TER than that observed at 11 h, despite a similar elevation in CH content (Fig. 4B).

TABLE 2
Protein and Cholesterol Content of Control and CH-MBCD–Treated MDCK Cell Monolayers[a]

CH added (µg/mL)	CH incubation time (h)	Protein (mg/insert)	CH (µg/insert)	µg CH/mg protein
0	11	0.61 ± 0.01	15.23 ± 0.64	24.91 ± 0.7
5	11	0.65 ± 0.01	17.44 ± 0.53	26.83 ± 0.31
10	11	0.65 ± 0.01	19.76 ± 0.35	30.64 ± 0.21
20	11	0.66 ± 0.01	19.97 ± 0.19	30.48 ± 0.18
0	24	0.52 ± 0.02	10.09 ± 0.03	19.53 ± 0.74
5	24	0.53 ± 0.01	13.12 ± 0.46	24.96 ± 0.26
10	24	0.50 ± 0.00	13.65 ± 0.42	27.19 ± 0.77
20	24	0.48 ± 0.01	14.75 ± 0.36	30.53 ± 0.83

[a]Data are expressed as the mean ± SEM of triplicate inserts. See Table 1 for abbreviations.

The cell-associated CH derived from exogenous CH-MBCD equilibrates with endogenous CH and is localized primarily to the plasma membrane. To compare the fraction of exogenous CH localized to the cells' surface with that of

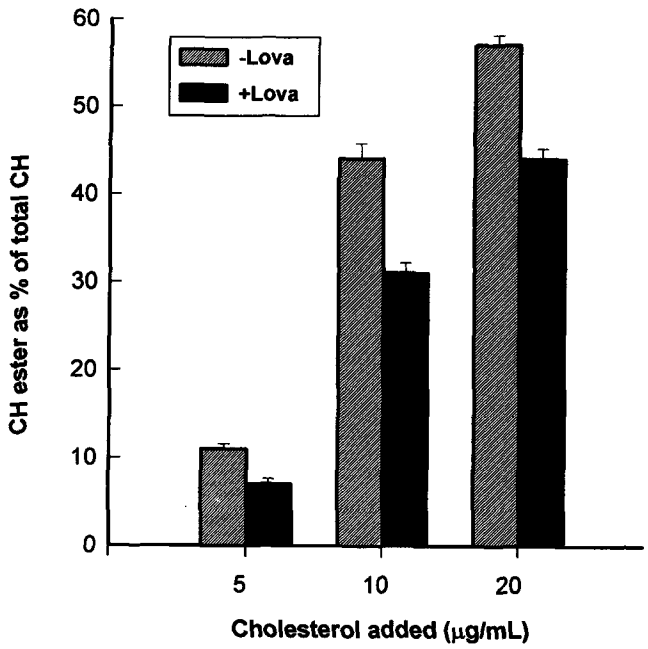


FIG. 5. CH ester synthesis from exogenous CH supplied to control and lova-treated MDCK cells. Cells were plated at confluence on inserts in Dulbecco's modified Eagle's medium/Ham's F-12, 10% BCS. Three hours later, they were transferred to low Ca^{2+} medium, 1% BCS, for 48 h. Half the monolayers were exposed to 2 µM lova for the last 24 h of this incubation while the remainder served as controls. At 37 h after plating the cells were incubated with 5, 10, or 20 µg/mL [^{14}C]CH as the CH-MBCD complex. Eleven hours later Ca^{2+}-sufficient medium, supplemented with the same concentration of [^{14}C]CH, was added. After 24 h of further incubation, monolayers were washed four times with phosphate-buffered saline, their lipids extracted, separated by thin-layer chromatography, and the radioactivity of the [^{14}C]CH and [^{14}C]CH ester spots were quantitated by liquid scintillation counting. Percent CH ester was calculated as: dpm [^{14}C]CH ester/(dpm [^{14}C]CH ester + dpm free [^{14}C]CH) × 100. Data are means ± SEM of triplicate inserts. See Figures 1 and 2 for abbreviations.

endogenous CH, CH oxidase, a probe for plasma membrane associated cholesterol, was employed (14,29). Monolayers in low Ca^{2+} medium (no formed TJ), that had been doubly labeled with [^{3}H]acetate (to label endogenous CH) and [^{14}C]CH from the MBCD complex, were incubated with CH oxidase after fixation with glutaraldehyde, and the fraction converted to radiolabeled cholestenone determined. When monolayers were not fixed with glutaraldehyde prior to incubation with CH oxidase, only 13% of the ^{3}H and ^{14}C in cell-associated CH was converted to cholestenone; pretreating with 1% glutaraldehyde increased this value to *ca.* 85% (Fig. 6). These data suggest that both endogenous CH and that taken up from the medium were equally accessible to CH oxidase. Further evidence that the added CH was an integral component of the plasma membrane was obtained by exposing mature monolayers, with formed TJ, to [^{14}C]CH-MBCD, added either to the apical or basolateral medium. Eleven hours later, the accessibility of the incorporated [^{14}C]CH to a solution of CH oxidase added to the opposite surface was determined. Within one hour of incubation at 37°C, approximately one-third of the incorporated [^{14}C]CH was oxidized to [^{14}C]cholestenone (Table 3), regardless of the cell surface that had been exposed to CH-MBCD or enzyme solution. This figure increased to 96% after six hours. That the TJ had retained their barrier function during the incubation was demonstrated by assaying the apical and basolateral solutions for CH oxidase activity one and six hours after addition of enzyme. No activity was detected after one hour, and at six hours the concentration of enzyme in the apical solution was only 5% of that in the basolateral solution.

The effect of exogenous CH on TER development by lova-treated monolayers may be mediated by mechanisms that compromise monolayer integrity. Cells were incubated with 5 μg/mL of CH during the last 11 h of a 24-h incubation with lova to determine the extent to which the time course for development of TER could be restored to that of controls. During the first five hours after addition of Ca^{2+} to the monolayer, the curve was only slightly below that of cells that had received only lova; beyond five hours, however, they diverged (Fig. 7A). By 12 h the TER values of monolayers incubated with both lova and CH overlapped those of controls and continued to do so for the remainder of the experiment. Raising the concentration of exogenous CH to 10 or 20 μg/mL diminished the rate and extent to which TER developed to well below that of controls, despite the fact that cell CH content did not increase beyond 30 μg/mg protein (Table 4). The protein content of these monolayers was virtually identical and no morphological differences between the monolayers was apparent upon examination by phase microscopy. If the incubation with lova and CH was extended to 24 h, virtually no

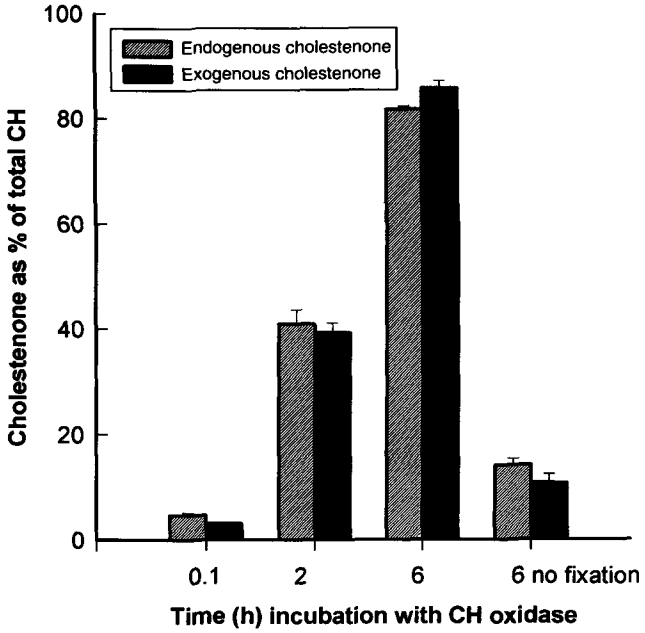


FIG. 6. Oxidation of cell-associated exogenous and endogenous CH to cholestenone with CH oxidase. Cells were plated at confluence on inserts at T_0 in Dulbecco's modified Eagle's medium/Ham's F-12, 10% BCS. Three hours later, they were incubated in low Ca^{2+} medium, 1% BCS, with 0.025 μCi/mL [^{3}H]acetate to label endogenous CH. After 37 h medium was changed to one containing 5 μg/mL [^{14}C]CH. Eleven hours later, monolayers were rinsed three times with mannitol buffer and all but one set fixed with 1% glutaraldehyde in mannitol buffer at 4°C for 15 min. Fixative was removed by rinsing three times with mannitol buffer. CH oxidase, 5 U/mL, was then added to both sides of the monolayer for 0.1, 2, and 6 h. Lipids were extracted and radiolabeled CH and cholestenone were isolated after separating them by thin-layer chromatography. Incorporated ^{3}H and ^{14}C were quantitated by liquid scintillation counting. Percent cholestenone was calculated as: dpm cholestenone/(dpm cholestenone + dpm CH) × 100. Data are means ± SEM of triplicate inserts. See Figures 1 and 2 for abbreviations.

TABLE 3
Cholesterol Taken Up from CH-MBCD at One Surface of the Cell Monolayer Is Accessible to Cholesterol Oxidase Added to the Other[a]

Side of [^{14}C]CH application[b]	Side of CH oxidase treatment	Time of oxidation (h)	CH (dpm)	Cholestenone (dpm)	% Oxidation of [^{14}C]CH
BL	AP	1	873	409	32
AP	BL	1	949	433	31
BL	AP	6	49	1200	96
AP	BL	6	52	1344	96

[a]Data are means of duplicate inserts. Duplicate values were within 5% of the mean. See Table 1 for abbreviations. [b]BL, basolateral; AP, apical.

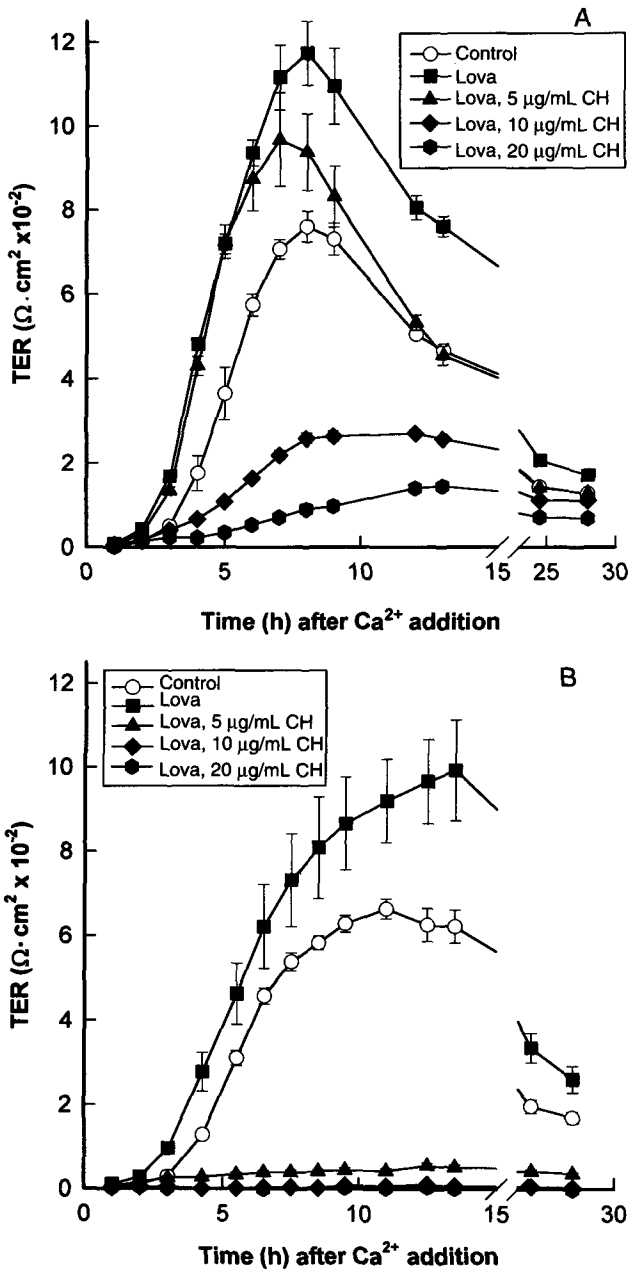


FIG. 7. Effect of exogenous CH on the development of TER in lova-treated cells relative to controls. Cells were plated on inserts as described in the legend to Figure 1. A: Twelve hours after addition of 2 µM lova, medium was changed to one containing the same concentration of lova and either 5, 10, or 20 µg/mL CH as the CH-MBCD complex. The same components were in the Ca^{2+}-sufficient medium, 1% BCS, used to initiate tight junction (TJ) formation. B: Cells were treated as in A, except that monolayers were exposed to 2 µM lova and 5, 10, or 20 µg/mL CH simultaneously 24 h before the addition of Ca^{2+}. TJ formation was initiated as described in A. Data points are means ± SEM of triplicate inserts. See Figures 1 and 2 for abbreviations.

TER developed after addition of Ca^{2+}, even at the lowest concentration of CH tested (Fig. 7B). In contrast to cultures incubated with either lova or CH alone, microscopic examination of these monolayers revealed that there were a greater number of cells floating, empty patches had formed, and the remaining adherent cells had become more round in appearance. Measurements of TER on such monolayers are not a valid measure of TJ formation. Such morphological changes have been observed in MDCK cells and other cell types exposed to much higher concentrations of lova (16,18–20). It is conceivable that exogenous CH down-regulated HMG–CoA reductase (22,33,34) and, by doing so, was acting in concert with lova to effectively reduce the level of intermediates between mevalonate and CH to a point that isoprenoid synthesis was impaired.

TABLE 4
Protein and Cholesterol Content of Control and Lovastatin-Treated MDCK Cell Monolayers Following the Administration of CH-MBCD[a]

CH added (µg/mL)	CH incubation time (h)	Protein (mg/insert)	CH (µg/insert)	µg CH/mg protein
0	11	0.62 ± 0.01	11.06 ± 0.23	17.74 ± 0.31
5	11	0.67 ± 0.01	17.23 ± 0.49	25.71 ± 0.81
10	11	0.68 ± 0.01	19.67 ± 0.56	28.97 ± 0.88
20	11	0.67 ± 0.01	19.86 ± 0.88	29.51 ± 0.58
0	24	0.52 ± 0.01	7.86 ± 0.24	15.08 ± 0.25
5	24	0.46 ± 0.01	10.89 ± 0.58	23.86 ± 0.63
10	24	0.14 ± 0.01	6.04 ± 0.20	39.69 ± 1.03
20	24	0.13 ± 0.00	5.98 ± 0.18	47.22 ± 1.48

[a]Data are expressed as the mean ± SEM of triplicate inserts. See Table 1 for abbreviations.

DISCUSSION

In an earlier study it was shown that exposing MDCK cells to 2 µM lova for 24 h resulted in a more rapid Ca^{2+}-induced development of TER, an index of TJ assembly (16). Several observations from that and other published studies suggested that lova's effect on TER were more closely related to changes in cell CH content than to changes in the level of prenyl group precursors. For example, the concentration of lova required to inhibit prenyl group synthesis is generally 10–15 times greater than that used in the current studies (18–20). Furthermore, only high concentrations (5 mM) of mevalonate were capable of overcoming lova's effects on TER; at 0.5 mM—a concentration of mevalonate which, in other cells, supports biosynthesis of isoprenoids (16,18,20)—lova's effects on TER were unaffected. This indirect evidence notwithstanding, there may be variations from one cell type to another in the sensitivity of isoprenoid biosynthesis to lova. One indirect measure of this variability, based on observations that indicate a role for isoprenoids in the regulation of cell growth (35), is the incorporation of [methyl-^{3}H]thymidine into DNA. Although 20–30 µM lova is required to interfere with protein prenylation in many cell types, it was recently shown that as little as 1 µM lova inhibited radiolabeled thymidine incorporation into DNA of kidney mesangial cells by mechanisms that involved depleting the intermediates required for prenyl group biosynthesis (36).

In the studies presented here, the relationship between cell CH content and development of TER were re-examined using

strategies that altered CH levels in a given direction by means that in some cases increased, and in others decreased, the flux of intermediates through that part of the pathway common to CH and isoprenoid biosynthesis. Zaragozic acid, by competitively inhibiting squalene synthase, decreased cell CH content and stimulated development of Ca^{2+}-induced TER to an extent similar to that caused by lova. This occurred despite the fact that, unlike lova, zaragozic acid has been shown to actually increase levels of intermediates required for prenyl group biosynthesis. After treatment of Hep G2 cells with zaragozic acid, for example, label from mevalonate accumulated in farnesyl pyrophosphate (32). In addition, labeling of prenylated proteins by [^{3}H]mevalonolactone was increased in livers from zaragozic acid-treated rats (21). The fact that both lova and zaragozic acid decreased cell CH and accelerated development of TER despite having opposing effects on the level of prenyl group intermediates, strongly suggests that their effects on TER are mediated by a fall in CH content. It should also be noted that in contrast to mesangial cells, any reduction in the level of prenylated proteins that might have been caused by lova were not sufficient to affect [methyl-^{3}H]thymidine incorporation into DNA. Control cultures and those incubated with 2 μM lova incorporated 5980 ± 330 DPM/mg protein and 6080 ± 20 DPM/mg protein, respectively.

To avoid prolonged incubation of cells with enzyme inhibitors altogether, MBCD, a water-soluble cyclodextrin that has been used for studies *in vivo* and *in vitro* to solubilize a variety of hydrophobic molecules, including CH (25,37), was employed. In a recent study cyclodextrins were shown to stimulate efflux of CH from fibroblasts and hepatoma cells (38). In the current study, MBCD at 2.0 mM stimulated sufficient CH efflux from MDCK cells to decrease cell CH to a degree similar to that achieved during longer incubations with lova or zaragozic acid. As was the case with those two inhibitors, monolayers treated with MBCD developed TER, after Ca^{2+} addition, more rapidly than the untreated controls. Reducing cell CH is known to increase HMG–CoA reductase activity (21,22,39) and should, therefore, maintain or even increase the flux of intermediates through that part of the CH biosynthetic pathway that supplies intermediates for prenyl group biosynthesis. This was confirmed by showing that [^{3}H]acetate incorporation into cell CH under these conditions was elevated by about 20% (Fig. 2). These data contrast with the effect of 2 μM lova, which decreased [^{3}H]acetate incorporation into CH by 27%. Interestingly, the increased incorporation of labeled acetate into CH caused by MBCD appears to be an adaptive response to the CH efflux. The CH mass after 2 h of incubation with 2 mM MBCD was reduced by 46%; after 24 h the cells had synthesized enough CH so that the mass was only 15% less than that of controls. Together these results suggest that it is the fall in cell CH and not a decrease in the availability of intermediates for prenyl group biosynthesis that accelerates the rate at which TER develops after Ca^{2+} addition.

If decreasing CH accelerates the rate at which TER develops in the confluent MDCK monolayer, then increasing cell CH might be expected to suppress it. This prediction was tested by incubating cells with a water-soluble complex of CH-MBCD. Incubation of cells for 11 h with 5, 10, and 20 μg/mL of CH as the CH-MBCD complex slowed the rate at which TER developed; the degree of decline was correlated with the increase in cell CH. A similar relationship between development of TER and increased cell CH was observed after a 24-h exposure of cells to CH-MBCD as well. At the lowest concentration of exogenous CH tested, only a small fraction of the CH taken up was converted to CH ester during 11 h of incubation. At 10 and 20 μg/mL this fraction increased markedly, indicating that the added CH was entering intracellular compartments and that the plasma membrane may be approaching saturation at relatively low levels of exogenous CH. A similar trend in CH ester formation was noted when lova-treated cells received exogenous CH. That there was a smaller percentage of the exogenous CH esterified by lova-treated cells is consistent with published data demonstrating that lova is an inhibitor of acyl-CoA:cholesterol acyltransferase (40). It should be noted that at the highest concentration of CH-MBCD used to increase cell CH, the total concentration of MBCD in the medium was only one-twentieth (0.09 mM) of that used to stimulate efflux. At that low concentration, in the presence of 1% BCS, there was no measurable decrease in cell CH, and the time course for development of TER was unaffected.

That the effects of incubation with CH-MBCD on TER are associated with incorporation of exogenous CH into the lipid bilayer of the plasma membrane is suggested by the following observations: (i) More than 85% of the cell CH, whether derived from endogenous or exogenous sources, was accessible to CH oxidase, a cell impermeant probe commonly employed in studies on the subcellular distribution of CH (29,41). (ii) Prior to fixation only 10–15% of the endogenous and exogenous CH pools were accessible to CH oxidase, a figure that agrees well with the accessibility of endogenous CH reported for fibroblasts (42) and LLC-PK1 cells (29). After fixation, on the other hand, 85–90% of both pools were enzymatically oxidized to cholestenone. The kinetics of the enzyme-catalyzed reaction were similar for both populations of radiolabeled CH. Although fixation with glutaraldehyde is necessary when CH oxidase is used as a probe (14,29,41), the chemical basis for this requirement is not known. It has been suggested, however, that glutaraldehyde, by reacting with amino groups of membrane components, may alter the organization of the cell surface, allowing greater access of the enzyme to its substrate (29). The fact that the enzymatic oxidation of both endogenous and exogenous CH pools is sensitive to glutaraldehyde suggests that the CH taken up from the CH-MBCD complex is not merely adsorbed to the cell surface, but is as integral to the membrane structure as endogenous CH. If it had been nonspecifically adsorbed to the cell surface, its accessibility to the enzyme would not be expected to increase after glutaraldehyde treatment. That 96% of the radiolabeled [^{14}C]CH taken up from one side of a mature mono-

layer with intact TJ was converted to cholestenone by CH oxidase added to the opposite compartment also supports the notion that once incorporated into one membrane domain (e.g., the apical surface), CH can move, by diffusional or transcytotic mechanisms, to the other (e.g., basolateral), where it is accessible to the enzyme.

Using CH-MBCD as a means of overcoming lova's effect on TER yielded data that were difficult to interpret. Exposing cells to CH at 5 μg/mL for 11 h did nothing to change lova's effect on TER development during the first five hours following Ca^{2+} addition. Beyond that time, the curve approached that of the untreated controls and overlapped it 12 h after Ca^{2+} addition. At higher concentrations of CH, under the same conditions, the time course was depressed well below that observed when only CH was added. None of the monolayers exposed to CH-MBCD for the entire 24 h of incubation with lova developed TER. Based on protein determinations and observations of those cultures by phase microscopy, however, it was clear that many cells had detached from the substratum and those that remained had become more rounded in appearance. Technically such changes would interfere with measurements of TER. It is not clear to what extent similar but more subtle changes in cell shape or monolayer integrity might have contributed to the results obtained after 11 h of incubation with both lova and CH-MBCD. Whatever the mechanism, it appears that exogenous CH, by raising cell CH, acts synergisticlly with lova, an agent that lowers it. This paradox may best be explained by focusing on how exogenous CH and lova each affect HMG–CoA reductase activity. Elevation of CH may down-regulate this enzyme (22,33,34) enough to slow CH synthesis without adversely affecting prenylation of proteins. When lova is also present simultaneously, however, any remaining activity is inhibited and mevalonate production slowed to such an extent that both CH synthesis and prenylation of proteins is decreased. Consistent with this explanation are data which show that as a percent of total lipid radioactivity, [^{3}H]acetate incorporation into CH declined from 47% (untreated) to 28, 16, and 7% in cultures that had been exposed for 24 h to 2 μM lova, 5 μg/mL CH, or both simultaneously. The rounding and detachment of cells that was observed in cultures incubated with both agents for 24 h was indistinguishable from that observed when cells were treated with 20–30 μM lova alone (16,18–20).

To summarize, measures that severely limit the availability of precursors required for prenylating proteins (e.g., incubations with both lova and CH) yield changes in monolayer integrity that make it difficult or technically impractical to use TER as a means of studying the formation of TJ. The data presented here do not rule out a role for prenylated proteins in TJ assembly, however, they do demonstrate that changes in cell CH can effectively modulate Ca^{2+}-induced TJ assembly by mechanisms that are independent of changes in the supply of intermediates required for protein prenylation.

How the Ca^{2+}-triggered assembly of TJ is modulated by CH is not clear. However, both CH and Ca^{2+} are known to affect the organization of membrane phospholipids as well as the activity of a variety of surface proteins (43–46), and the initial interaction with Ca^{2+} apparently occurs at a point on, or very close to, the plasma membrane (47). A change in CH, among other things could: (i) Affect the generation of signals from the plasma membrane that initiate TJ assembly (11,48); (ii) alter membrane fusion events (10) and, as a consequence, the traffic of surface components needed for TJ; (iii) promote or retard the interactions between the plasma membrane and cytoskeletal elements (49,50) or between membrane proteins of adjacent cells. This latter possibility is of particular interest in view of a recent study which indicates that removal of plasma membrane CH causes a reduction in bilayer thickness and, conversely, addition of CH increases its thickness (51). It is possible that homotypic interactions between surface elements (e.g., occludin) leading to TJ assembly between adjacent epithelial cells are facilitated when membrane width decreases by removal of CH or hampered if it increases upon CH incorporation. Additional studies are in progress to determine which of the many events linking Ca^{2+} binding at the cell surface to assembly of TJ are sensitive to changes in CH content.

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Effect of Dietary n-9 Eicosatrienoic Acid on the Fatty Acid Composition of Plasma Lipid Fractions and Tissue Phospholipids

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ABSTRACT: n-9 Eicosatrienoic acid (ETrA), also known as Mead acid, is a minor fatty acid in essential fatty acid (EFA)-sufficient healthy subjects but is found at increased levels in EFA deficiency. This study examined the influence of dietary ETrA from a biological source on plasma and tissue ETrA. A synthetic fat-free diet was prepared to which was added Mut 48 oil which contains 19% ETrA (wt%) as well as other n-9 fatty acids. Blends of vegetable oils were used to achieve overall diets with 5% fat (wt%) and varying amounts of ETrA at two different dietary levels of linoleic acid (LA), approximately 4.4 and 19% of total fatty acids. These diets were fed to 5-week-old Dark Agouti rats for four weeks. Plasma lipid fractions and liver, spleen, and peritoneal exudate (PE) cells were analyzed for fatty acid composition. ETrA was present at up to 20% total fatty acids in plasma triglyceride, cholesterol ester, and phospholipid fractions. ETrA also accumulated to substantial levels in phospholipids of liver and spleen (up to 15% of total fatty acids) and PE cells (up to 11%). ETrA was found in plasma and tissue phospholipids in proportion to the amount of ETrA present in the diet. The incorporation was reduced in diets with higher LA content compared to diets containing similar amounts of ETrA but lower LA. All rats remained apparently healthy, and histological survey of major organs revealed no abnormality. While the long-term implications for health of ingestion of diets rich in ETrA remain to be established, rats appear to tolerate high levels of dietary ETrA without adverse effects. Dietary enrichment with ETrA warrants further investigation for possible beneficial effects in models of inflammation and autoimmunity, as well as in other conditions in which mediators derived from n-6 fatty acids can affect homeostasis adversely.
Lipids 31, 829–837 (1996).

Dietary n-6 polyunsaturated fatty acids (PUFA) are present in the Western diet at levels far in excess of essential requirements (1). As a result, arachidonic acid (AA; 20:4n-6) is the dominant substrate for eicosanoid-forming enzymes which are pivotal in platelet-vascular homeostasis and control of inflammation. Consequently, the balance of eicosanoids produced is prothrombotic and proinflammatory as evinced by the antithrombotic and antiinflammatory effects of agents such as aspirin, which inhibit prostaglandin H synthase. These effects of n-6 eicosanoids can be reduced by dietary fortification with the n-3 homologue of AA, eicosapentaenoic acid (EPA; 20:5n-3). This fatty acid competes for eicosanoid-forming enzymes, and is metabolized to products, which on balance, reduce risk for thrombotic events and modulate inflammation.

Dietary fortification with the n-9 homologue of AA and EPA, eicosatrienoic acid (ETrA; 20:3n-9), also can antagonize n-6 eicosanoid formation (2). However, the lack of a suitable biological source of ETrA has been a barrier to experimental feeding studies. The present study examines the effects of Mut 48 oil, an ETrA-rich oil derived from a strain of the fungus *Mortierella alpina*, on the fatty acid composition of plasma lipid fractions and cellular phospholipids in rats. Because accumulation of EPA in plasma and cell phospholipids is reduced by dietary linoleic acid (LA; 18:2n-6), ETrA accumulation was assessed at two levels of dietary LA (3). ETrA is a normal metabolite which is typically present at low levels in plasma and cells, except in the setting of n-6 essential fatty acid (EFA) deficiency when levels increase. Evidence for signs of the EFA deficiency syndrome (coat changes and growth retardation) were, therefore, sought in rats fed the ETrA-fortified diets.

METHODS

Materials. Mortierella alpina oil was obtained from Suntory Ltd. (Osaka, Japan). Sunflower oil and Sunola™ were obtained from Meadow Lea Foods Ltd. (Sydney, Australia). Sunola is an oil obtained from a sunflower strain which yields an oil with a low LA content (~4.4% total fatty acids) compared to regular sunflower oil which contained approximately 70% LA.

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Abbreviations: AA, arachidonic acid; EFA, essential fatty acid; EPA, eicosapentaenoic acid; ETrA, n-9 eicosatrienoic acid; LA, linoleic acid; PE cells, peritoneal exudate cells, PUFA, polyunsaturated fatty acids.

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TABLE 1
Fatty Acid Composition of Diets[a]

	Dietary groups[b]								
Fatty acids	16:4	12:4	12:19	8:4	8:19	4:4	4:19	0:4	0:19
Total saturated	22.6	19.8	20.3	16.4	17.2	13.3	13.9	10.0	10.7
Totals *trans*	0.2	0.1	0.1	0.3	0.1	0.3	0.3	0.4	0.3
18:1n-9	34.4	47.1	31.5	59.6	43.6	71.8	56.3	84.0	68.6
Total monosaturated	38.4	50.4	34.9	62.2	46.3	73.6	58.2	85.1	69.7
18:2n-9	13.7	10.1	10.3	6.7	7.0	3.4	3.5	—	0.2
20:2n-9	2.5	1.9	1.9	1.3	1.2	0.6	0.6	—	—
20:3n-9	16.5	12.2	12.2	8.1	8.1	4.0	4.0	—	—
22:3n-9	0.2	0.1	0.2	0.1	0.1	—	—	—	—
Total n-9	68.4	72.3	56.9	76.3	60.6	80.1	64.8	84.0	68.8
18:2n-6	4.6	4.4	19.2	4.4	19.3	4.4	19.1	4.4	18.9
Total n-6	5.2	4.9	19.7	4.7	19.6	4.5	19.2	4.4	18.9
Total n-3	0.9	0.6	0.7	0.4	0.5	0.3	0.4	0.2	0.3

[a]Expressed as percentage of total fatty acids as determined by gas–liquid chromatography analysis.
[b]The test diets are designated according to the ratios of their approximate content of linoleic acid and n-9 eicosatrienoic acid.

Diets. A set of oil mixtures containing approximately 12, 8, or 4% (wt%) of ETrA in combination with LA at levels of 4.4 and 19% (wt%) were prepared by using varying amounts of sunflower, Sunola and *M. alpina* oils. In a further preparation, *M. alpina* oil was blended with a small amount of sunflower oil to yield an oil mix containing 16.5% (wt%) of ETrA and 4.4% (wt%) of LA. In addition, Sunola alone and a blend of Sunola with sunflower oil were used to achieve oils with undetectable ETrA and 4.4 or 19% (wt%) of LA, respectively. These preparations were mixed with a fat-free diet which was based on the AIN-76 diet, as published previously (4). The total fat content of the final diet was 5% (wt%). The salient details of the test diets in relation to fatty acids of interest are detailed in Table 1. The full fatty acid analysis is detailed elsewhere (5).

Rats. Female Dark Agouti rats were used with groups of four rats being fed the test diets from five weeks of age for four weeks. The rats were observed and given fresh food daily, and weighed weekly. Peritoneal exudates (PE) were induced with peptone 3% (g/100 mL) four hours prior to sacrifice as described previously (4).

Collection of samples. Rats were sacrificed by cervical dislocation under halothane/nitrous oxide anesthesia. Blood samples were taken by cardiac puncture. PE cells were obtained by lavage as described (4). The liver, spleen, kidney, heart, and skin specimens were excised. The study was

TABLE 2
Plasma Phospholipid Fatty Acids[a]

	Dietary group[b]								
Fatty acids	16:4	12:4	12:19	8:4	8:19	4:4	4:19	0:4	0:19
Total saturated	45.4 ± 0.2	46.2 ± 0.4	48.1 ± 0.6	45.9 ± 0.2	47.8 ± 1.1	48.3 ± 0.9	48.3 ± 0.6	46.1 ± 1.0	47.8 ± 0.7
Total monosaturated	10.0 ± 0.4	10.1 ± 0.3	9.1 ± 0.4	12.8 ± 0.4	10.2 ± 1.1	13.2 ± 0.8	11.1 ± 1.1	17.2 ± 1.8	12.4 ± 0.3
18:2n-9	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
20:2n-9	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	n.d.[c]
20:3n-9	20.2 ± 1.3	17.2 ± 1.4	6.6 ± 0.5	14.4 ± 1.3	4.2 ± 0.2	8.5 ± 0.7	2.7 ± 0.4	3.0 ± 0.8	0.3 ± 0.0
22:3n-9	0.7 ± 0.0	0.7 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	n.d.
Total n-9	28.3 ± 1.3	25.5 ± 1.4	14.0 ± 0.9	24.5 ± 1.1	11.9 ± 1.2	19.0 ± 0.8	11.4 ± 1.2	16.7 ± 2.2	10.2 ± 0.2
18:2n-6	4.0 ± 0.1	4.0 ± 0.3	7.0 ± 0.9	4.5 ± 0.2	5.8 ± 0.4	4.7 ± 0.3	7.3 ± 0.6	6.1 ± 0.5	7.0 ± 0.5
20:3n-6	0.6 ± 0.0	0.7 ± 0.0	0.6 ± 0.1	0.8 ± 0.1	0.6 ± 0.0	1.0 ± 0.1	0.7 ± 0.1	1.3 ± 0.2	0.6 ± 0.1
20:4n-6	13.5 ± 0.1	15.2 ± 1.1	21.7 ± 1.3	15.6 ± 0.9	24.6 ± 0.7	18.6 ± 0.8	23.3 ± 1.2	20.1 ± 2.2	25.2 ± 0.5
22:4n-6	0.2 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.7 ± 0.0
22:5n-6	0.5 ± 0.0	0.7 ± 0.1	1.0 ± 0.2	0.7 ± 0.1	1.3 ± 0.2	1.0 ± 0.1	1.6 ± 0.3	1.3 ± 0.1	1.9 ± 0.3
Total n-6	18.9 ± 0.8	21.0 ± 1.0	31.0 ± 0.5	22.0 ± 0.7	33.0 ± 0.5	25.6 ± 0.6	33.7 ± 0.6	29.3 ± 1.3	35.6 ± 0.8
20:5n-3	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	n.d.
22:5n-3	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
22:6n-3	3.6 ± 0.3	3.6 ± 0.4	3.5 ± 0.2	3.1 ± 0.1	3.5 ± 0.2	3.1 ± 0.2	2.8 ± 0.4	3.0 ± 0.4	2.7 ± 0.2
Total n-3	4.4 ± 0.3	4.3 ± 0.2	4.1 ± 0.3	3.9 ± 0.2	3.9 ± 0.2	3.6 ± 0.2	3.2 ± 0.4	3.5 ± 0.4	3.0 ± 0.2

[a]Expressed as percentage of total fatty acids; mean ± SD, $n = 4$ rats in each group. [b]n-9 Eicosatrienoic acid/linoleic acid in diet expressed as percentage of total fatty acids. [c]n.d. = Not detected.

TABLE 3
Plasma Triglyceride Fatty Acids[a]

	Dietary group[b]								
Fatty acids	16:4	12:4	12:19	8:4	8:19	4:4	4:19	0:4	0:19
Total saturated	27.8 ± 2.1	28.7 ± 0.7	21.1 ± 1.0	22.5 ± 0.8	23.9 ± 1.8	26.3 ± 0.7	24.1 ± 1.1	25.4 ± 0.7	25.7 ± 0.6
Total monosaturated	39.1 ± 1.0	41.7 ± 0.7	33.4 ± 1.9	47.7 ± 3.4	36.7 ± 1.5	55.0 ± 2.0	45.2 ± 1.5	62.6 ± 3.2	51.1 ± 2.1
18:2n-9	2.6 ± 0.3	2.0 ± 0.3	2.5 ± 1.2	1.6 ± 0.2	1.2 ± 0.1	1.8 ± 0.4	1.5 ± 0.6	1.3 ± 0.4	2.4 ± 0.7
20:2n-9	1.4 ± 0.1	0.9 ± 0.2	1.0 ± 0.3	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.0	n.d.[c]	n.d.
20:3n-9	22.4 ± 2.5	20.2 ± 0.8	22.7 ± 2.3	19.3 ± 1.6	15.3 ± 1.8	8.5 ± 1.8	8.8 ± 1.8	2.4 ± 0.3	1.0 ± 0.0
22:3n-9	1.3 ± 0.2	0.9 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	n.d.
Total n-9	58.5 ± 2.3	58.7 ± 1.0	54.5 ± 1.3	62.9 ± 1.6	48.5 ± 2.0	58.9 ± 1.1	49.8 ± 1.3	58.3 ± 2.5	48.3 ± 1.9
18:2n-6	4.1 ± 0.6	3.8 ± 0.2	11.7 ± 0.9	4.6 ± 0.5	11.6 ± 0.5	4.8 ± 0.3	11.3 ± 1.2	4.7 ± 0.9	11.0 ± 0.9
20:3n-6	0.1 ± 0.1	n.d.	0.2 ± 0.1	n.d.	0.2 ± 0.0	n.d.	0.2 ± 0.2	0.1 ± 0.1	0.2 ± 0.2
20:4n-6	1.6 ± 0.2	1.9 ± 0.4	5.2 ± 1.2	1.9 ± 0.7	8.0 ± 0.8	2.5 ± 0.2	6.2 ± 1.3	2.7 ± 1.3	6.7 ± 1.2
22:4n-6	0.1 ± 0.1	n.d.	0.4 ± 0.1	n.d.	0.5 ± 0.1	0.1 ± 0.2	0.7 ± 0.2	0.2 ± 0.2	0.9 ± 0.2
22:5n-6	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	n.d.	0.4 ± 0.0	0.1 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.6 ± 0.1
Total n-6	6.0 ± 0.7	6.0 ± 0.5	18.2 ± 0.6	6.6 ± 1.1	21.2 ± 1.2	7.6 ± 0.8	19.5 ± 0.5	8.0 ± 2.4	19.7 ± 1.9
20:5n-3	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	n.d.	0.3 ± 0.1	n.d.	0.1 ± 0.1	n.d.	n.d.
22:5n-3	0.1 ± 0.1	n.d.	n.d.	n.d.	0.1 ± 0.1	n.d.	n.d.	n.d.	n.d.
22:6n-3	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.1	0.2 ± 0.3
Total n-3	0.8 ± 0.2	0.5 ± 0.1	0.9 ± 0.1	0.5 ± 0.2	0.9 ± 0.2	0.4 ± 0.2	0.6 ± 0.1	0.3 ± 0.1	0.2 ± 0.3

[a]Expressed as percentage of total fatty acids; mean ± SD, $n = 4$ rats in each group. [b]n-9 Eicosatrienoic acid/linoleic acid in diet expressed as percentage of total fatty acids. [c]n.d. = Not detected.

approved by the Animal Ethics Committee of the Institute of Medical and Veterinary Science (Adelaide, Australia) where the animals were housed.

Fatty acid analyses. Lipids were extracted from plasma or homogenized tissue samples with chloroform/methanol (7:3), and extracts were evaporated to dryness under nitrogen. The residues were dissolved in chloroform/methanol (9:1) and stored at −70°C prior to fatty acid analysis. Plasma triglycerides, cholesterol esters, and phospholipids and tissue phospholipid fractions were separated by thin-layer chromatography (6). The samples were then hydrolyzed and methylated in 1% H_2SO_4 and methanol at 70°C for three hours. The resulting methyl esters were separated and quantified by gas–liquid chromatography as described previously (7). All organic solvents contained butylated hydroxyanisole 0.005% (g/100 mL).

Table 4
Plasma Cholesterol Ester Fatty Acids[a]

	Dietary group[b]								
Fatty acids	16:4	12:4	12:19	8:4	8:19	4:4	4:19	0:4	0:19
Total saturated	9.3 ± 1.1	7.1 ± 1.1	6.2 ± 0.3	6.1 ± 0.1	5.4 ± 0.4	6.0 ± 0.2	5.5 ± 0.4	5.6 ± 0.2	5.4 ± 0.1
Total monounsaturated	11.9 ± 0.9	10.3 ± 0.4	8.1 ± 0.4	13.0 ± 0.1	8.1 ± 0.1	13.7 ± 0.6	9.3 ± 1.4	16.2 ± 2.0	10.1 ± 0.7
18:2n-9	2.4 ± 0.1	1.7 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.5 ± 0.1
20:2n-9	0.2 ± 0.0	0.1 ± 0.1	0.1 ± 0.2	0.1 ± 0.0	n.d.	n.d.	n.d.	n.d.	n.d.
20:3n-9	24.9 ± 2.1	22.1 ± 1.6	7.0 ± 0.6	17.9 ± 1.7	4.5 ± 0.3	9.9 ± 0.5	2.8 ± 0.4	3.3 ± 1.1	0.3 ± 0.0
22:3n-9	0.1 ± 0.0	n.d.[c]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total n-9	36.4 ± 2.1	31.8 ± 2.1	14.6 ± 0.9	29.2 ± 1.6	11.4 ± 0.2	21.7 ± 1.0	10.5 ± 1.4	16.8 ± 2.5	9.1 ± 0.6
18:2n-6	4.7 ± 0.9	5.1 ± 0.4	8.1 ± 0.6	5.6 ± 0.4	7.2 ± 0.5	5.8 ± 0.3	8.8 ± 0.5	7.6 ± 0.9	8.8 ± 0.6
20:3n-6	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.6 ± 0.1	0.3 ± 0.0
20:4n-6	42.8 ± 2.4	49.8 ± 3.5	65.8 ± 1.3	52.4 ± 2.3	71.1 ± 1.1	60.8 ± 1.2	70.4 ± 2.5	63.7 ± 4.1	72.6 ± 1.0
22:4n-6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22:5n-6	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.1
Total n-6	48.4 ± 2.4	55.9 ± 3.2	75.2 ± 1.2	59.2 ± 1.9	79.5 ± 0.6	67.7 ± 1.0	80.5 ± 2.1	72.8 ± 3.1	82.6 ± 0.6
20:5n-3	1.1 ± 0.1	1.0 ± 0.1	0.3 ± 0.0	1.0 ± 0.1	0.3 ± 0.0	0.6 ± 0.1	0.2 ± 0.0	0.7 ± 0.1	0.2 ± 0.0
22:5n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22:6n-3	1.4 ± 0.1	1.4 ± 0.1	1.3 ± 0.0	1.2 ± 0.0	1.2 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.0 ± 0.1
Total n-3	2.5 ± 0.1	2.4 ± 0.1	1.7 ± 0.2	2.2 ± 0.1	1.5 ± 0.1	1.8 ± 0.1	1.2 ± 0.1	1.9 ± 0.0	1.1 ± 0.1

[a]Expressed as percentage of total fatty acids; mean ± SD, $n = 4$ rats in each group. [b]n-9 Eicosatrienoic acid/linoleic acid in diet expressed as percentage of total fatty acids. [c]n.d. = Not detected.

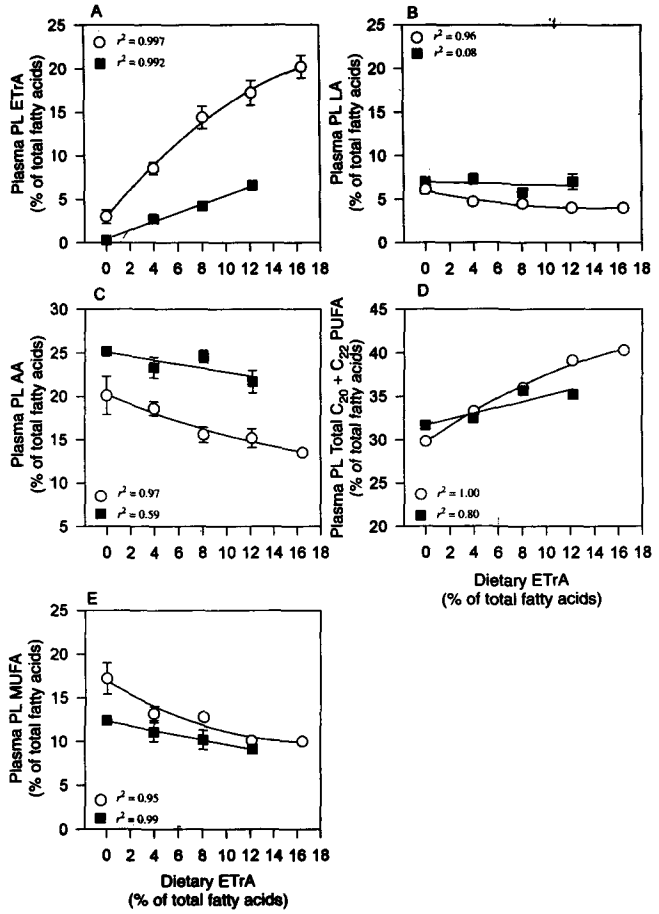


FIG. 1. Plasma phospholipids (PL): Levels of selected fatty acids in plasma lipid fractions from rats fed for four weeks diets containing linoleic acid (LA) at 4.4 % of total fatty acids (O----O) or LA at 19% of total fatty acids (■-----■) and increasing amounts of n-9 eicosatrienoic acid (ETrA). A, ETrA; B, LA; C, arachidonic acid (AA); D, total C_{20} + C_{22} polyunsaturated fatty acids (C_{20} +C_{22} PUFA); E, monounsaturated fatty acids (MUFA).

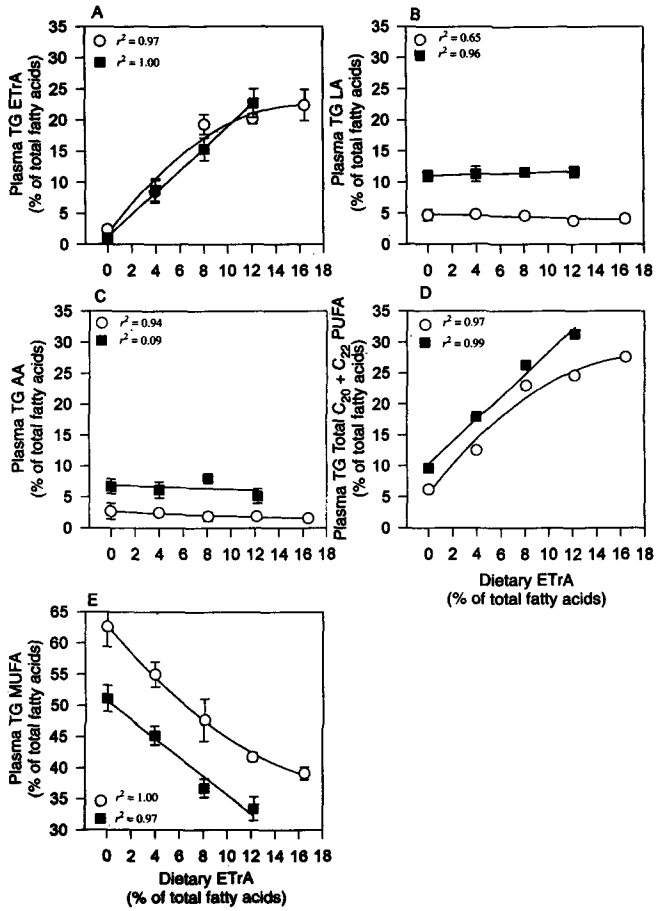


FIG. 2. Plasma triglycerides (TG): See Figure 1 caption for data.

Histological survey. Formalin fixed sections of liver, spleen, kidney, skin, and heart were stained with haematoxalin and eosin and examined by light microscopy.

RESULTS

Fatty acid composition of plasma lipid fractions (Tables 2–4 and Figs. 1–3). With the diets containing the lower level of LA (4.4% total fatty acids), ETrA attained levels of ~20% total fatty acids in all of the plasma lipid fractions, and incorporation was curvilinear in relation to dietary ETrA. Within the ambit of levels attained when the diets contained the higher level of LA (19% total fatty acids), incorporation of ETrA was essentially linear. In the phospholipid and cholesterol ester fractions, but not the triglycerides, ETrA accumulation was reduced substantially by dietary LA.

Accumulation of LA was greater with the higher LA diets than with the lower LA diets, although the differences were modest, except in the triglycerides. By contrast, AA accumulation was strongly influenced by dietary LA in the case of the plasma phospholipids and cholesterol esters. The levels of LA were modestly affected or unaffected by dietary ETrA. However, with increasing dietary ETrA, dose-dependent reductions of AA were seen in all fractions with the exception of the triglycerides where AA levels were low. With increasing dietary ETrA, total C_{20} and C_{22} PUFA increased by up to 10% in the phospholipids and by more than 20% in the triglycerides (with reciprocal displacement of monounsaturates) but were unchanged in the cholesterol esters.

Fatty acid composition of cellular phospholipids (Tables 5–7 and Figs. 4–6). The effects of dietary ETrA and LA on the levels of ETrA, LA, and AA in liver, spleen, and PE cell phospholipids resembled broadly the pattern seen in plasma phospholipids. Compared with liver and plasma phospholipids, spleen and PE cell phospholipids were distinctive in displaying significant accumulations of 22:3n-9, a metabolite of ETrA absent or present in trace amounts only in the diets. With increasing dietary ETrA, there was an overall increase in C_{20} and C_{22} PUFA as a proportion of total fatty acids, with an increment of ~10% of total fatty acids for spleen and PE cells and ~5% of total fatty acids for liver. In liver and spleen, but not PE cells, monounsaturates decreased with increasing dietary ETrA.

General observations and growth. All rats appeared normal in terms of general behavior and condition. Growth was

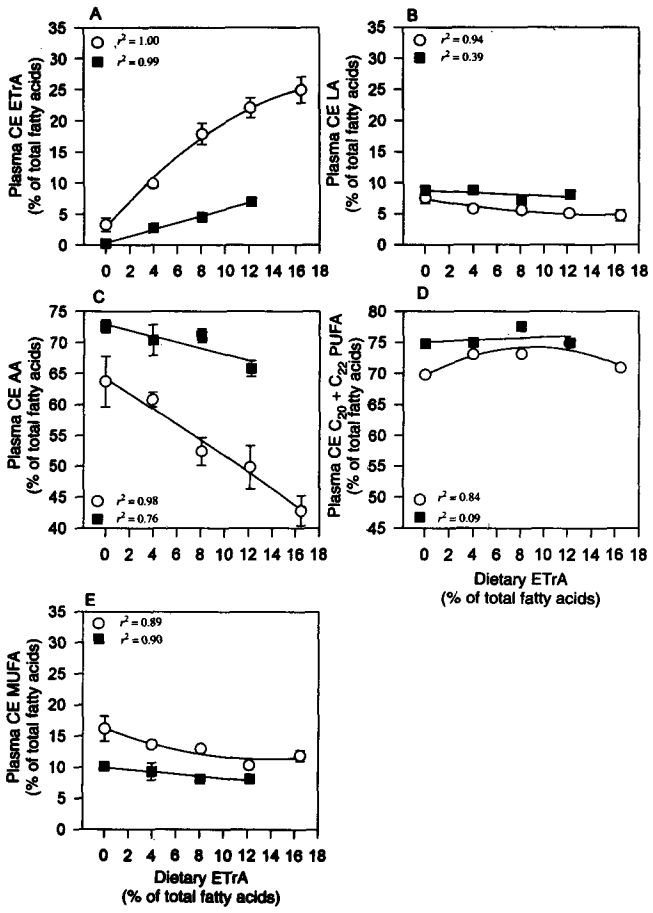


FIG. 3. Plasma cholesterol esters (CE): See Figure 1 caption for data.

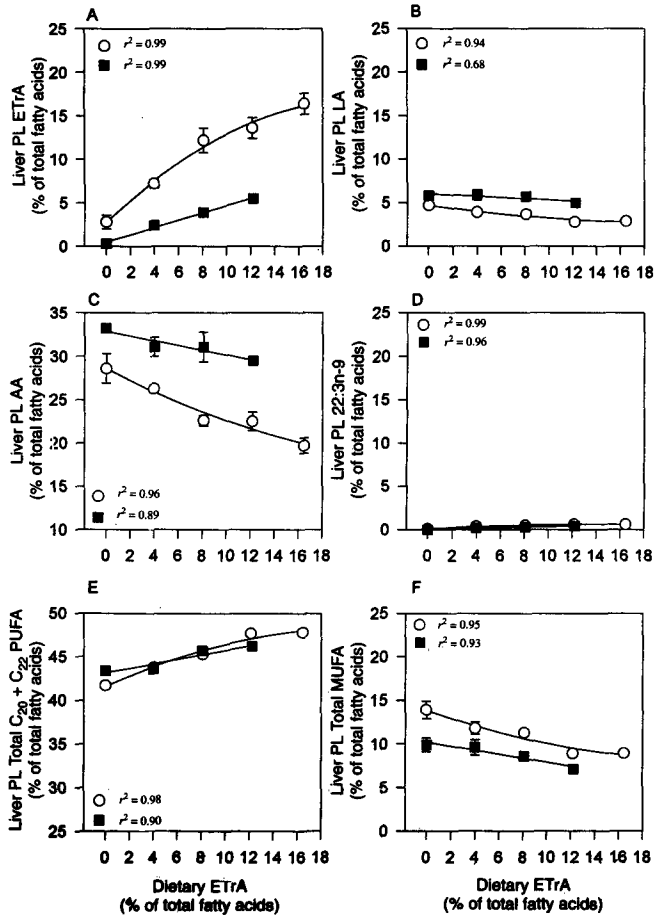


FIG. 4. Liver: Levels of selected fatty acids in tissue and cell phospholipid extracts from rats fed for four weeks diets containing LA at 4.4 % of total fatty acids (O----O) or LA at 19% of total fatty acids (■-----■) and increasing amounts of ETrA. A, ETrA; B, LA; C, AA; D, n-9 docosatrienoic acid (22:3n-9); E, total C_{20} + C_{22} PUFA; F, MUFA. See Figure 1 for abbreviations.

accelerated in rats receiving ETrA relative to rats receiving the diets with no ETrA, regardless of the LA content. There appeared to be a direct relationship between dietary ETrA and weight gain (Fig. 7).

Histological survey. All organs and tissues examined histologically (liver, spleen, kidney, heart, and skin) displayed normal appearances.

DISCUSSION

These investigations show that dietary ETrA appears prominently in plasma lipid fractions and that it is incorporated efficiently into cellular phospholipids in a dose-dependent manner. Levels of ETrA attained 20% of total fatty acids in each of the plasma fractions and 11–16% in PE cell, spleen, and liver. Our data suggest that both plasma fractions and cell membrane phospholipids are saturable in relation to ETrA. The facility with which ETrA accumulates suggests that it competes favorably with potential fatty acid competitors for enzymes responsible for acylation of fatty acids into cell membranes and plasma phospholipids.

Dietary LA had an important negative effect on incorporation of dietary ETrA into plasma lipid fractions and membrane phospholipids. For a given dietary intake of ETrA, levels of ETrA in phospholipids from rats fed the higher LA diets were only 25 to 30% of the values attained with the lower diets. We have reported similar effects of dietary LA on incorporation into plasma lipid fractions and cell membranes of EPA, the n-3 homologue of ETrA (3).

The major n-9 PUFA incorporated into all the plasma fractions and tissues examined was ETrA despite the fact that the diets contained a range of n-9 PUFA. In particular, 18:2n-9 (~14%) was only slightly less abundant in the Mut 48 oil than 20:3n-9 (~16%). That the level of 18:2n-9 only once exceeded 2% total fatty acids in any fraction tested suggests that metabolism of this species occurs readily. One possible route of metabolism of 20:2 n-9 is desaturation by δ5 desaturase to ETrA, which, in contrast to δ6 desaturase, does not appear to be rate-limiting in relation to desaturation of PUFA (8).

In rats, ETrA can be metabolized further to 22:3n-9 (2). This fatty acid was present in only trace amounts in the Mut 48 oil-containing diets and was found in only small amounts (<1% fatty acids) in the plasma fractions and liver phospholipids. By contrast, more substantial accumulation of 22:3n-9

TABLE 5
Liver Phospholipid Fatty Acids[a]

Fatty acids	Dietary group[b]								
	16:4	12:4	12:19	8:4	8:19	4:4	4:19	0:4	0:19
Total saturated	40.1 ± 0.4	40.4 ± 0.2	40.9 ± 0.2	39.1 ± 1.2	38.7 ± 1.8	39.7 ± 0.6	39.9 ± 0.5	38.6 ± 0.3	39.6 ± 0.7
Total monounsaturated	8.9 ± 0.0	8.9 ± 0.4	7.1 ± 0.3	11.3 ± 0.4	8.6 ± 0.5	11.8 ± 0.7	9.6 ± 0.9	13.9 ± 1.0	9.9 ± 0.8
18:2n-9	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	1.1 ± 0.3	0.6 ± 0.2	0.5 ± 0.2	0.7 ± 0.1	0.7 ± 0.2
20:2n-9	0.5 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	n.d.[c]	n.d.
20:3n-9	16.4 ± 1.2	13.6 ± 1.2	5.5 ± 0.4	12.2 ± 1.4	3.9 ± 0.2	7.2 ± 0.5	2.4 ± 0.4	2.8 ± 0.8	0.3 ± 0.0
22:3n-9	0.6 ± 0.0	0.6 ± 0.1	0.4 ± 0.1	0.5 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	n.d.
Total n-9	22.9 ± 1.2	20.1 ± 1.2	10.7 ± 0.4	20.3 ± 1.1	10.3 ± 0.4	15.7 ± 0.7	9.0 ± 1.2	12.7 ± 1.6	7.2 ± 0.6
18:2n-6	2.9 ± 0.1	2.8 ± 0.1	5.0 ± 0.1	3.7 ± 0.4	5.7 ± 0.3	3.9 ± 0.2	5.9 ± 0.6	4.7 ± 0.3	5.8 ± 0.4
20:3n-6	0.7 ± 0.0	0.8 ± 0.0	0.7 ± 0.1	1.0 ± 0.1	0.7 ± 0.0	1.1 ± 0.1	0.9 ± 0.1	1.4 ± 0.2	0.8 ± 0.1
20:4n-6	19.7 ± 0.9	22.5 ± 1.1	29.5 ± 0.3	22.6 ± 0.6	31.1 ± 1.7	26.3 ± 0.4	31.1 ± 1.1	28.6 ± 1.7	33.2 ± 0.2
22:4n-6	0.2 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.7 ± 0.1
22:5n-6	0.7 ± 0.0	1.1 ± 0.1	1.5 ± 0.2	1.0 ± 0.1	1.8 ± 0.3	1.4 ± 0.1	2.3 ± 0.3	1.8 ± 0.2	2.7 ± 0.5
Total n-6	24.2 ± 1.0	27.5 ± 1.0	37.5 ± 0.2	28.7 ± 0.6	40.1 ± 1.8	33.2 ± 0.2	41.1 ± 0.5	37.2 ± 1.0	43.6 ± 0.5
20:5n-3	0.5 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.4 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0
22:5n-3	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
22:6n-3	8.0 ± 0.4	7.7 ± 0.5	7.3 ± 0.3	6.8 ± 0.3	6.7 ± 0.6	6.5 ± 0.2	5.5 ± 0.5	6.0 ± 0.6	5.3 ± 0.5
Total n-3	9.0 ± 0.4	8.5 ± 0.5	7.9 ± 0.4	7.5 ± 0.3	7.2 ± 0.6	7.0 ± 0.2	6.0 ± 0.5	6.5 ± 0.6	5.7 ± 0.5

[a]Expressed as percentage of total fatty acids; mean ± SD, $n = 4$ rats in each group. [b]n-9 Eicosatrienoic acid/linoleic acid in diet expressed as percentage of total fatty acids. [c]n.d. = Not detected.

was observed in spleen and PE cell. This accumulation displayed a highly significant ($r^2 > 0.99$) second-order correlation with dietary ETrA with apparent saturation at levels of 4% for spleen and ~7% for PE cells. Reciprocal depression of the n-6 homologue 22:4n-6 was seen. Metabolism of dietary ETrA differs from EPA (and to a lesser extent AA) with regard to the lack of evident accumulation of the n-9 homologue of n-3 docosahexaenoic acid (DHA) in PE cells (2).

The accomodation of ETrA within plasma phospholipids and cell membranes was achieved by combinations of displacement of AA and monounsaturates and expansion of the sum contribution of C_{20} and C_{22} fatty acids. The differences in the patterns of accumulation of ETrA and other fatty acids between different plasma lipid fractions highlight the shortcomings of analyses confined to total plasma lipids. For example, in the cholesterol esters, an essentially reciprocal relationship between ETrA and AA levels was seen without the increase of $C_{20} + C_{22}$ fatty acids seen with the plasma phospholipids and triglycerides. As discussed above, with spleen and PE cell phospholipids, the ETrA metabolite C22:3n-9

TABLE 6
Spleen Phospholipid Fatty Acids[a]

Fatty acids	Dietary group[b]								
	16:4	12:4	12:19	8:4	8:19	4:4	4:19	0:4	0:19
Total saturated	37.6 ± 0.2	37.7 ± 0.2	38.7 ± 0.3	39.8 ± 0.0	40.7 ± 0.5	39.6 ± 0.2	40.3 ± 0.7	38.7 ± 0.5	39.9 ± 0.1
Total monounsaturated	20.3 ± 0.3	21.8 ± 0.4	18.3 ± 0.2	22.7 ± 0.4	18.4 ± 0.4	23.9 ± 0.4	19.3 ± 0.1	25.3 ± 0.4	20.4 ± 0.3
18:2n-9	1.8 ± 0.6	1.2 ± 0.2	1.2 ± 0.4	1.2 ± 0.2	1.5 ± 0.6	1.2 ± 0.1	1.7 ± 0.9	2.0 ± 1.0	1.2 ± 0.1
20:2n-9	1.8 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.0 ± 0.1	0.9 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	n.d.[c]
20:3n-9	15.2 ± 1.0	13.1 ± 0.5	7.6 ± 0.2	10.1 ± 0.5	5.1 ± 0.2	6.0 ± 0.1	3.1 ± 0.0	1.5 ± 0.2	0.3 ± 0.2
22:3n-9	4.1 ± 0.1	3.9 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	1.6 ± 0.0	0.6 ± 0.1	0.2 ± 0.0
Total n-9	34.3 ± 0.8	32.5 ± 0.5	23.5 ± 0.5	29.8 ± 0.8	21.0 ± 0.7	25.9 ± 0.4	19.7 ± 0.9	22.7 ± 1.5	16.5 ± 0.3
18:2n-6	3.4 ± 0.1	3.3 ± 0.1	6.4 ± 0.1	3.2 ± 0.1	5.9 ± 0.1	3.3 ± 0.1	6.1 ± 0.2	3.4 ± 0.1	6.0 ± 0.0
20:3n-6	0.7 ± 0.0	0.7 ± 0.0	1.1 ± 0.0	0.8 ± 0.0	1.0 ± 0.0	0.9 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.1 ± 0.0
20:4n-6	16.4 ± 0.7	17.8 ± 0.4	21.5 ± 0.3	18.2 ± 0.5	22.3 ± 0.3	21.0 ± 0.3	23.1 ± 0.3	23.2 ± 1.0	25.2 ± 0.2
22:4n-6	0.9 ± 0.1	1.1 ± 0.0	1.8 ± 0.0	1.3 ± 0.1	2.1 ± 0.1	1.8 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	3.4 ± 0.1
22:5n-6	0.2 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.7 ± 0.1	0.7 ± 0.0	0.9 ± 0.1
Total n-6	21.8 ± 0.8	23.4 ± 0.5	31.7 ± 0.5	24.1 ± 0.5	32.4 ± 0.3	27.6 ± 0.3	34.0 ± 0.3	31.0 ± 1.0	37.2 ± 0.2
20:5n-3	0.2 ± 0.0	0.1 ± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22:5n-3	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.2	0.3 ± 0.0
22:6n-3	1.0 ± 0.0	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.0	0.7 ± 0.1	1.0 ± 0.1	0.8 ± 0.1
Total n-3	1.6 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.5 ± 0.3	1.2 ± 0.1

[a]Expressed as percentage of total fatty acids; mean ± SD, $n = 4$ rats in each group. [b]n-9 Eicosatrienoic acid/linoleic acid in diet expressed as percentage of total fatty acids. cn.d. = Not detected.

TABLE 7
Peritoneal Exudate Cell Phospholipid Fatty Acids[a]

	Dietary group[b]								
Fatty acids	16:4	12:4	12:19	8:4	8:19	4:4	4:19	0:4	0:19
Total saturated	36.4 ± 0.3	35.9 ± 0.8	36.6 ± 0.2	41.2 ± 0.9	41.7 ± 1.1	39.7 ± 1.5	40.9 ± 0.3	39.2 ± 0.5	40.5 ± 1.4
18:1n-9	10.5 ± 0.4	11.5 ± 0.6	9.8 ± 0.4	11.8 ± 0.8	10.0 ± 0.4	13.6 ± 0.6	11.2 ± 1.3	14.6 ± 1.3	12.4 ± 0.3
Total monounsaturated	23.2 ± 0.7	24.0 ± 0.9	18.9 ± 0.2	22.6 ± 0.6	18.1 ± 0.4	23.8 ± 1.8	19.8 ± 0.7	22.6 ± 0.9	18.9 ± 1.1
18:2n-9	3.7 ± 1.1	3.9 ± 1.1	3.9 ± 0.7	5.3 ± 1.9	6.1 ± 2.9	5.2 ± 4.9	5.7 ± 2.2	6.2 ± 1.0	5.8 ± 3.9
20:2n-9	2.2 ± 0.1	1.9 ± 0.1	1.5 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	0.5 ± 0.3	n.d.	0.1 ± 0.3	n.d.
20:3n-9	11.3 ± 0.6	9.3 ± 0.4	5.3 ± 0.0	6.7 ± 0.7	3.5 ± 0.2	4.0 ± 0.2	1.9 ± 0.1	1.0 ± 0.2	n.d.
22:3n-9	6.7 ± 0.3	6.6 ± 0.4	4.2 ± 0.2	4.9 ± 0.2	3.1 ± 0.3	2.8 ± 0.8	1.8 ± 0.1	0.7 ± 0.5	n.d.
Total n-9	36.5 ± 1.2	35.3 ± 0.8	26.4 ± 0.8	32.0 ± 1.1	25.6 ± 2.9	29.0 ± 4.2	23.6 ± 1.5	25.9 ± 1.3	20.9 ± 3.9
18:2n-6	2.8 ± 0.1	2.7 ± 0.2	5.1 ± 0.3	2.2 ± 0.1	4.4 ± 0.1	2.4 ± 0.2	4.4 ± 0.7	2.6 ± 0.3	4.6 ± 0.3
20:3n-6	0.7 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.1	0.8 ± 0.0	0.6 ± 0.4	0.9 ± 0.1	1.0 ± 0.1	0.7 ± 0.5
20:4n-6	17.0 ± 1.2	17.7 ± 1.0	22.1 ± 0.5	16.6 ± 0.4	19.6 ± 1.6	19.4 ± 0.7	20.5 ± 2.9	20.6 ± 1.5	21.6 ± 0.5
22:4n-6	2.5 ± 0.2	3.2 ± 0.3	4.2 ± 0.2	3.4 ± 0.5	4.5 ± 0.3	3.8 ± 0.8	5.0 ± 1.3	5.3 ± 1.1	7.0 ± 0.4
22:5n-6	n.d.	0.1 ± 0.2	0.4 ± 0.3	n.d.	0.2 ± 0.3	0.4 ± 0.3	0.8 ± 0.2	0.5 ± 0.4	0.7 ± 0.5
Total n-6	22.9 ± 1.4	24.5 ± 1	32.6 ± 0.9	23.0 ± 0.4	29.6 ± 2.0	26.7 ± 1.2	31.6 ± 2.5	30.1 ± 1.1	34.7 ± 1.7
20:5n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22:5n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22:6n-3	0.4 ± 0.4	0.5 ± 0.3	0.3 ± 0.3	0.1 ± 0.2	0.0 ± 0.0	0.2 ± 0.3	0.2 ± 0.3	0.1 ± 0.2	0.1 ± 0.2
Total n-3	0.4 ± 0.4	0.5 ± 0.3	0.3 ± 0.3	0.1 ± 0.2	0.0 ± 0.0	0.2 ± 0.3	0.2 ± 0.3	0.1 ± 0.2	0.1 ± 0.2

[a]Expressed as percentage of total fatty acids; mean ± SD, $n = 4$ rats in each group. [b]n-9 Eicosatrienoic acid/linoleic acid in diet expressed as percentage of total fatty acids. [c]n.d. = Not detected.

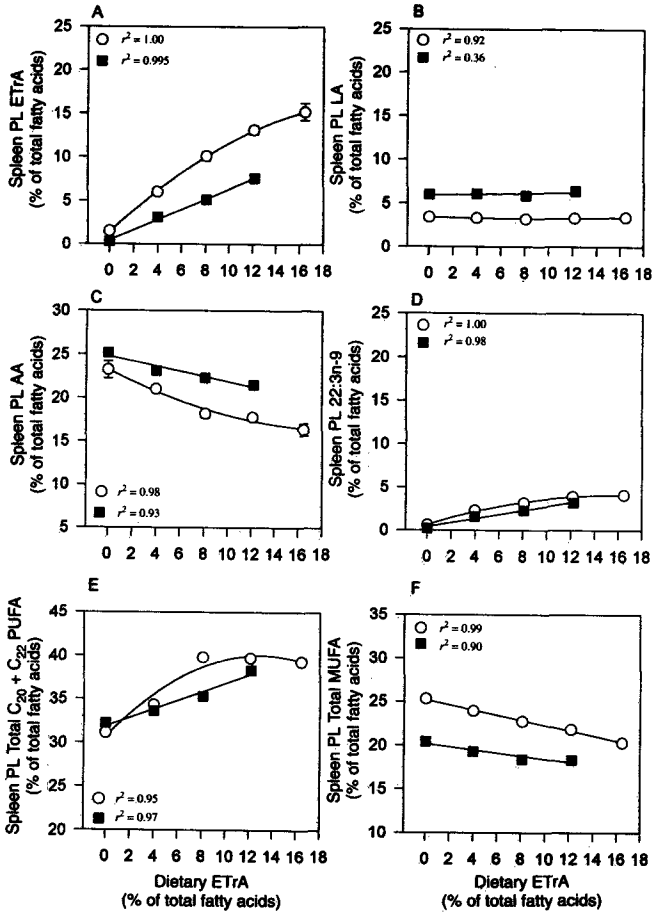


FIG. 5. Spleen: See Figure 4 caption for data.

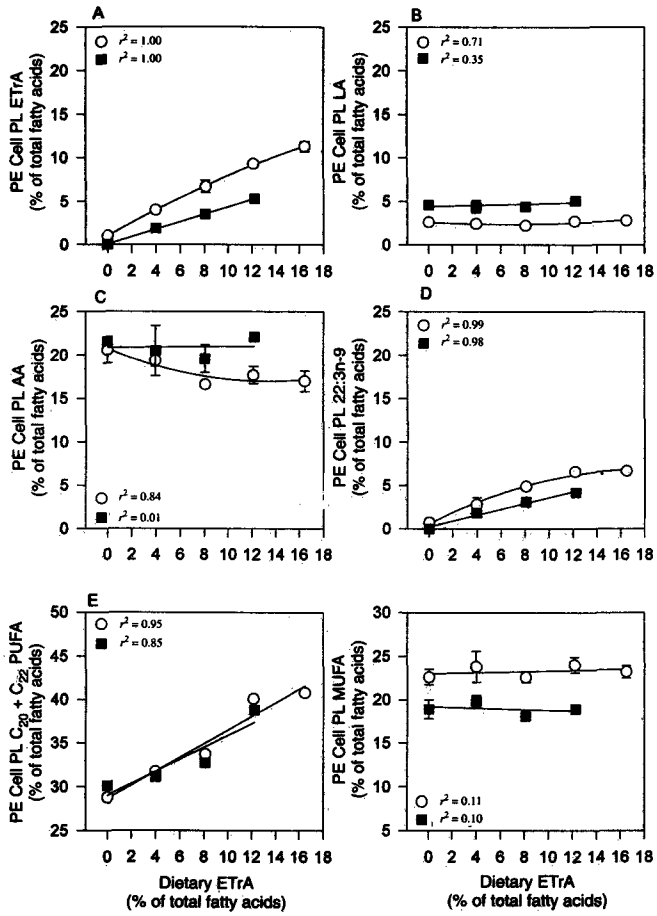


FIG. 6. Peritoneal exudate cells (PE cell): See Figure 4 caption for data.

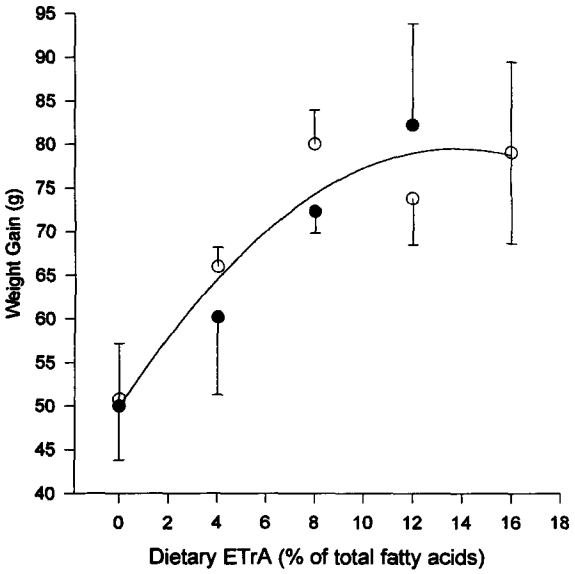


FIG. 7. Mean weight gain (g) observed in groups of rats ($n = 4$) during a four-week period of feeding with diets containing LA at 4.4% of total fatty acids (O----O) or LA at 19% of total fatty acids (■-----■) and increasing amounts of ETrA. See Figure 1 for abbreviations.

also accumulated. The accumulations of ETrA and 22:3n-9 in cells appeared to be potentially saturable as evinced by the observed hyperbolic relationships with dietary ETrA.

With the exception of the plasma cholesterol ester fraction, plasma and cellular AA were only partially displaced by dietary ETrA (as discussed above). This is similar to changes seen in EFA deficiency where AA levels are relatively conserved in the presence of LA depletion and ETrA accumulation (9). The ETrA-rich diets were associated with a modest reduction in LA in some, but not all, fractions or tissues. With the exception of the plasma triglycerides, dietary LA had little effect on LA accumulation in the various fractions, thus establishing the EFA-sufficiency of the lower LA diets. The salient biochemical point of distinction between the effects of ETrA-rich diets and EFA deficiency is the profound LA depletion in plasma and cells seen only in the latter. This is an important difference since the loss of cutaneous barrier function seen in EFA deficiency has been attributed directly to LA depletion (10).

The positive effect on growth of the ETrA-rich diets suggests that ETrA, or some other factor within Mut 48 oil, possesses general metabolic effects. However, only group data with large variation are available from this experiment, and further investigation on weight gain in individual rats is needed to confirm this apparent finding. Nevertheless, the present studies at least establish that dietary ETrA has no negative effects on growth *per se*, in contrast to the reported effects of EFA deficiency.

The very substantial incorporation of ETrA from the diet into cells observed raises questions as to the effects of this incorporation on cell metabolism. As a C_{20} PUFA, ETrA is a potential substrate competitor with AA for eicosanoid-forming enzymes. However, ETrA cannot be converted to a cyclic eicosanoid, such as a thromboxane or prostaglandin, because it lacks the n-6/δ14 double bond. Nevertheless, ETrA could influence eicosanoid synthesis through displacement of AA at the *sn*-2 position of phospholipids, resulting in reduced release of AA by phospholipases. This effect would be analogous to that seen with long-chain n-3 fatty acids where the antiinflammatory effects of purified EPA and DHA ethyl esters in a model sensitive to cyclooxygenase inhibition (carrageenan-induced paw oedema in the rat) correlated with the extent of reduction of AA as a proportion of total C_{20} and C_{22} PUFA in leucocyte phospholipids (11).

In a separate component of the present study, we analyzed the effects of the test diets on leukotriene B_4 (LTB_4) production by rat PE cells stimulated *in vitro*. Dietary ETrA inhibited LTB_4 production in a dose-dependent manner, and the effect was attributable to dose-dependent inhibition of LTA hydrolase (5). These observations confirm and extend our earlier studies into the effects of dietary supplementation with synthetic ETrA on LTB_4 production by leucocytes in rats (2), and are also consistent with *in vitro* studies into the effects of exogenous ETrA on LTB_4 production (9,12). The substantial incorporation of ETrA seen in spleen cells raises the possibility that dietary fortification of ETrA may have other important immunomodulatory effects. It is also possible that 22:3n-9, which accumulates in spleen and PE cell phospholipids, could also have immunomodulatory actions.

A clue to possible beneficial effects of ETrA may be found in the homology between ETrA and EPA, a constituent of fish oils which has been shown to have antiinflammatory effects (13,14). Conceivably, future investigations could be undertaken to assess the effects of ETrA in animal models of human diseases and, in particular, diseases in which there is an immunological pathogenesis or an important inflammatory component. The expected greater stability of ETrA-rich oils relative to n-3 fatty acid-rich oils, which have immunomodulatory effects, makes dietary ETrA fortification potentially a more acceptable and perhaps more effective strategy. If ETrA proves to be nontoxic and useful in reducing the incidence and/or severity of inflammatory diseases in susceptible animals, dietary studies could be undertaken in humans, involving, in the first instance, those with immunoinflammatory disorders or those identified as being at increased risk.

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Vitamin E Inhibits Fish Oil-Induced Hyperlipidemia and Tissue Lipid Peroxidation in Hamsters

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ABSTRACT: Previous research has linked hyperlipidemia with increased serum concentrations of lipid peroxidation products; however, a specific association between diet-induced oxidative stress and hyperlipidemia has not been studied. In the present study, the relationship between tissue lipid peroxidation and hyperlipidemia induced by ingestion of fish oil was examined. In Experiment 1, male Golden Syrian hamsters were fed semi-purified diets composed of 1.6 wt% safflower oil plus 15.0 wt% of either butterfat (BF), safflower oil (SAFF), or high-cholesterol menhaden oil [MHO(H-CHOL)] semipurified diets for 27 d. The cholesterol contents of the diets were adjusted to 0.088%. The MHO(H-CHOL)-fed hamsters exhibited higher serum concentrations of total cholesterol, triglycerides, apolipoprotein B, and lipid peroxides when compared to the BF and SAFF diet groups. In a further study (Experiment 2), hamsters were fed for 27 d three dietary treatments: (i) MHO(H-CHOL) with no vitamin E content; (ii) a low-cholesterol menhaden oil containing high concentrations of vitamin E (2.5 mg tocopherol/g oil or dietary concentrations of 375 mg/kg) [MHO(L-CHOL) + E]; and (iii) the MHO(L-CHOL + E) with added cholesterol (595 mg/kg) [MHO(L-CHOL) + CHOL + E] to match the cholesterol content of the MHO(H-CHOL). The MHO(L-CHOL) + E and MHO(L-CHOL) + CHOL + E diet groups showed lower concentrations of serum cholesterol, triglycerides, and hepatic lipid peroxides than the MHO(H-CHOL)-treated group. Moreover, in contrast to the hypercholesterolemia caused by the MHO(H-CHOL) feeding, the MHO(L-CHOL)+ E and MHO(L-CHOL) + CHOL + E diets did not show a serum cholesterol-elevating action. This study supports the hypothesis that oxidative stress in the Syrian hamster could play a causal role in dietary-induced hyperlipidemia which can be inhibited by high vitamin E intake.
Lipids 31, 839–847 (1996).

Lipid peroxidation and oxidative stress have been associated with the occurrence of hypercholesterolemia and atherosclerosis both in humans and experimental animals (1–5). Despite associations of oxidative stress with hyperlipidemia, a possible relationship between diet-induced oxidative stress and diet-induced hyperlipidemia has not been closely examined.

Fish-oil feeding generally results in reductions in plasma triglyceride and very low density lipoprotein concentrations (6), but there does exist conflicting research. Investigations have shown elevated serum triglycerides in the New Zealand rabbit (7) and the Golden Syrian hamster (8) following fish-oil feeding. In addition, the fish oil-fed Syrian hamster had an elevated apolipoprotein B/apolipoprotein AI ratio (8), which may be correlated with an increased risk of atherosclerosis and may be a better predictor of coronary heart disease than plasma lipid concentrations *per se* (9). Fish-oil concentrates given to hypercholesterolemic men have caused increased plasma total and low density lipoprotein (LDL) cholesterol concentrations (10).

Fish oils rich in n-3 polyunsaturated fatty acids (PUFA) that are efficiently incorporated into tissue membrane phospholipids displacing n-6 PUFA, and membranes enriched in n-3 fatty acids are likely to be more susceptible to oxidative disruption (11). Dietary fish-oil consumption has been shown to increase aortic lesions in rabbits and cause higher plasma levels of lipid peroxidation products as measured by the production of thiobarbituric acid reactive substances (TBARS) (12). In addition, oxidative stress generated by fish-oil consumption is known to increase the requirement of vitamin E (12). As lipid peroxidation (1) and a low body content of tocopherol (5) have been shown to increase tissue cholesterol concentrations, the accompanying increase in oxidative stress could play a causal role. Indeed, the ability of fish oil to decrease plasma triglyceride concentrations in the normolipidemic human has been shown to be markedly enhanced by high supplemental intake of vitamin E (13). Haglund *et al.* (13) suggested that the antioxidant effect of vitamin E was involved through efficient inhibition of fatty acid peroxidation, thereby allowing larger amounts of active n-3 fatty acids in fish oils to exert triglyceride-lowering effects.

In the present study, two experiments were performed with the Syrian hamster. In Experiment 1, the relationship between tissue concentrations of lipid peroxidation products and serum lipid concentrations was investigated among menhaden

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Abbreviations: ANOVA, analysis of variance; BF, butterfat; HDL, high density lipoprotein; LDL, low density lipoprotein; MHO(H-CHOL), high-cholesterol menhaden oil; MHO(L-CHOL) + E, low-cholesterol menhaden oil with a high vitamin E content; MHO(L-CHOL) + CHOL + E, low-cholesterol menhaden oil with a high vitamin E content and added cholesterol; NRC, National Research Council; PUFA, polyunsaturated fatty acids; SAFF, safflower oil; TBARS, thiobarbituric acid reactive substances; USP, United States Pharmacopeia.

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oil-, butterfat (BF), and safflower oil (SAFF)-based diets. In contrast to fish oils, SAFF feeding was not expected to increase tissue lipid peroxidation as tissue concentrations of lipid peroxidation products have previously been shown not to be increased by high n-6 PUFA intake as compared to high-saturated fatty acid intake (14). Dietary studies comparing the effects of different fat sources on blood cholesterol metabolism have typically not balanced for the vitamin E content nascent in each fat. Hence, in the initial experiment, the effects of the different fat sources on blood cholesterol metabolism and lipid peroxidation were assessed without balancing for the vitamin E content as this could inhibit the effects of fish oil on lipid peroxidation and on blood lipid concentrations. In a second experiment, we tested whether a high intake of vitamin E could modify the dietary cholesterol-induced hyperlipidemia and tissue lipid peroxidation associated with fish oil-based diets.

MATERIALS AND METHODS

Experimental design. In Experiment 1, hamsters were fed semipurified diets containing 1.6 wt% SAFF and 15 wt% of either BF, SAFF, or a high cholesterol (5.76 mg cholesterol/g of oil) menhaden oil [MHO(H-CHOL)] (Table 1). In experiment 1, the effects of these different fat sources on blood cholesterol metabolism and lipid peroxidation were examined without matching for vitamin E content as additional vitamin E might have compromised effects of MHO(H-CHOL) on lipid peroxidation and on blood lipid concentrations. The MHO(H-CHOL) and BF had nondetectable concentrations of vitamin E, whereas SAFF contained 0.42 g vitamin E/g oil (Table 1). Sufficient vitamin E (6.1 mg vitamin E/kg diet) was added to the BF and MHO(H-CHOL) semipurified diets to meet the National Research Council (NRC) requirements for adequate vitamin E intake for the hamster (15). As BF con-

TABLE 1
Composition of Experimental Diets (g/kg)[a]

Ingredients	BF	SAFF	MHO(H-CHOL)	MHO(L-CHOL) + E + CHOL	MHO(L-CHOL) + E
Egg white	150	150	150	150	150
Cornstarch	538	538	538	538	538
Butterfat[b]	178	—	—	—	—
Safflower oil[c]	16	166	16	16	16
Menhaden oil[d]	—	—	150	150	150
Cholesterol[e]					
Naturally occurring	0.44	—	0.88	0.285	0.285
Added	0.44	0.88	—	0.595	—
Total	0.88	0.88	0.88	0.88	0.285
Cellulose	50	50	50	50	50
Vitamin mix[f]	14	14	14	14	14
Mineral mix[g]	80	80	80	80	80

[a]BF = butterfat; SAFF = safflower oil, MHO(H-CHOL) = high cholesterol (5.76 mg cholesterol/g oil) menhaden oil; MHO(L-CHOL) + CHOL + E = low cholesterol (1.9 mg cholesterol/g oil) MHO with high vitamin E content (2.5 mg vitamin E/g oil) and added cholesterol (595 mg/kg); MHO(L-CHOL) + E = low cholesterol (1.9 mg cholesterol/g oil) MHO with high vitamin E content (2.5 mg vitamin E/g oil).

[b]Nondetectable concentrations of vitamin E in BF. Fatty acid composition of BF is as follows (mg%): 4:0, 3.3%; 6:0, 2.3%; 8:0, 1.6%; 10:0, 3.9%; 12:0, 4.0%; 14:0,12.5%; 14:1, 1.2%; 16:0, 33.1%; 16:1n-9, 1.7%; 18:0, 11.6%; 18:1n-9, 21.0%; 18:2n-6, 3.8%.

[c]In Experiments 1 and 2, all diets contained a minimum of 1.6 wt% SAFF to prevent essential fatty acid deficiency. Vitamin E concentration of SAFF is 0.42 g/g oil. Fatty acid composition of SAFF is as follows (mg%): 14:0, 0.3%; 16:0, 11.9%; 18:0, 2.3%; 18:1n-9, 14.1%; 18:2n-6, 70.9%; 18:3n-3, 0.5%.

[d]Nondetectable concentrations of vitamin E in MHO(H-CHOL) and 2.5 mg vitamin E/g oil in MHO(L-CHOL). Fatty acid composition of H-CHOL MHO is as follows (mg%): 14:0, 15.2%; 16:0, 22.3%; 16:1n-9, 12.7%; 18:0, 3.9%; 18:1n-9, 10:0%; 18:2n-6, 2.8%; 18:3n-3, 1.1%; 18:4n-3, 4.5%; 20:1n-9, 1.4%; 20:4n-6, 0.5%; 20:5n-3, 12.7%; 22:1n-9, 1.3%; 22:4n-6, 0.5%; 22:5n-3, 1.5%; 22:6n-3, 9.6%. Fatty acid composition of MHO(L-CHOL) is as follows (mg%): 14:0, 7.8%; 16:0, 14.7%; 16:1n-9, 0.2%; 18:0, 2.7%; 18:1n-9, 5.2%; 18:2n-6, 1.4%; 18:3n-3, 0.9%; 18:4n-3, 2.5%; 20:1n-9, 0.8%; 20:4n-6, 0.8%; 20:5n-3, 13.6%; 22:1n-9, 0.2%; 22:4n-6, 0.2%; 22:5n-3, 2.5%; 22:6n-3, 10.2%.

[e]Cholesterol United States Pharmacopeia (USP) was added to the oil, BF, and MHO(L-CHOL) diets at 880, 440, and 595 (mg/kg diet), respectively. No cholesterol was added to the MHO(H-CHOL) diet because it naturally contains 5.76 mg cholesterol/g of oil. BF naturally contains 2.39 mg cholesterol/g BF, MHO(L-CHOL) contains 1.9 mg cholesterol/g oil, and SAFF contains no cholesterol.

[f]Vitamin mix supplied the following (mg/kg diet): niacin, 135; calcium pantothenate, 60; riboflavin, 22.5; pyridoxine, 9; thiamin, 30; folic acid, 3; biotin, 0.9; cyanocobalamine, 0.015; menaquinone, 6; choline, 6000; α-tocopheryl acetate (1350 IU/g), 6.1; cholecalciferol (400,000 IU/g), 9.32; vitamin A palmitate (250,000 IU/g), 40.2. Only 18.5 mg/kg of vitamin A palmitate (250,000 IU/g) was provided to the BF diets since vitamin A occurs naturally in BF at a level of 30.07 IU/g.

[g]Mineral mix supplied the following (mg/kg diet): calcium carbonate, 22.48; potassium phosphate, 25.16; sodium chloride, 5.72; magnesium sulfate, 4.46; cupric carbonate, 0.0045; cobalt chloride, 0.0666; sodium fluoride, 0.00008; potassium iodate, 0.0040; ferric sulfate, 1.045; manganous carbonate, 0.0115; sodium selenite, 0.00033; zinc carbonate, 0.0265.

tained 2.39 mg cholesterol/g and SAFF contained no cholesterol, United States Pharmacopeia (USP) cholesterol was added to the SAFF and BF diets at 880 and 440 mg (mg/kg diet), respectively, to match levels in the MHO(H-CHOL). In Experiment 2, hamsters were fed one of three semipurified diets which consisted of: (i) the high-cholesterol MHO(H-CHOL); (ii) a low-cholesterol menhaden oil which also contained high amounts of vitamin E (2.5 mg vitamin E/g oil) equivalent to a dietary concentration of 375 mg/kg [MHO(L-CHOL) + E]; and (iii) the low-cholesterol MHO(L-CHOL) + E oil (2.5 mg vitamin E/g oil) which was supplemented with USP cholesterol (595 mg/kg diet) [MHO(L-CHOL) + CHOL + E] to match the high-cholesterol content found in MHO(H-CHOL).

Animals and diets. Male Syrian hamsters, weighing 110–120 g, were purchased at seven weeks of age from Charles River Inc. (St-Constant, Quebec, Canada). The animals were fed a commercial nonpurified diet (Mouse Chow; Purina, Montreal, Quebec, Canada) upon arrival. After an adaptation period of three weeks, the hamsters received semipurified diets for 27 d in two separate experiments. Thirty-six hamsters were weighed and randomly assigned to three semipurified diet groups in Experiment 1, whereas in Experiment 2, twenty-one hamsters were weighed and allocated randomly to three semipurified diet groups. In Experiments 1 and 2, all diets contained minimally sufficient vitamin E (6.1 mg vitamin E/kg diet) to meet the NRC requirements of vitamin E for the hamster (15). In all diets, butylated hydroxytoluene was added to dietary fat sources at a level of 0.1% of lipid weight to protect fats from oxidation. The MHO(H-CHOL) was purchased from U.S. Biochemical Corporation (Cleveland, OH), and the MHO(L-CHOL) was a gift from the Fish Oil Biomedical Test Material Program of the National Marine Fisheries Service (Washington, D.C.). The differences in fatty acid content between the MHO(L-CHOL) and MHO(H-CHOL) oils as indicated in Table 1 were minimal, i.e., total n-3 content (mg %) = 29.4 [MHO(L-CHOL)] and 29.7 [MHO(H-CHOL)]; n-6/n-3 ratio = 0.09 [MHO(L-CHOL)] and 0.12 [MHO(H-CHOL)]; unsaturation indices = 167 [MHO(L-CHOL)] and 184 [MHO(H-CHOL)]; eicosapentaenoic acid (20:5n-3)/arachidonic acid (20:4n-6) ratio = 17 [MHO(L-CHOL)] and 25.4 [MHO(H-CHOL)]; 20:5n-3/docosahexaenoic acid (22:6n-3) ratio = 1.3 [MHO(H-CHOL)] and 1.3 [MHO(L-CHOL)]. Based on previous literature, the magnitude of the differences in fatty acid composition would not be expected to impact upon serum lipid concentrations (16,17). Unused fresh diets were kept in plastic bags at 4°C, and bags were flushed with nitrogen after each opening during the trial. Samples of stored semipurified diet were measured for TBARS on a weekly basis throughout the experiment to obtain an index of dietary lipid peroxidation by a modification (18) of the method of Dahle *et al.* (19). Analysis of all semipurified diets yielded nondetectable values of TBARS, e.g., below 20 nmol of reference standard (malondialdehyde *bis*diethyl acetal). Suitable vitamin and mineral mixes for hamster needs were developed following the NRC requirements (15) and were given at 1.5 times the requirements to ensure an adequate intake of those nutrients. BF (Lactancia, Victoriaville, Quebec, Canada) contained 18.4% water (manufacturer's specifications). This factor was taken into account in the formulation of the BF diet (e.g., 177.6 g/kg of BF was added into the diet instead of 150.0 g/kg of lipid that was added in the other diets) in order to make the diets isoenergetic.

The animals were housed individually in plastic bottom wired top cages and kept in a temperature-controlled room (23–25°C) with a 12-h automatically maintained light-dark cycle (light cycle 0700 to 1900). They were allowed access to food and water *ad libitum*. Fresh diets were fed to the animals daily, and uneaten food was discarded. Body weight was measured every second day, and food intake was recorded daily. Care and handling of the animals conformed to the guidelines of the McGill University Animal Care Committee and Canadian Council of Animal Care (20).

Tissue sampling and preparation. On day 1 and day 27, blood was collected using heparinized capillary tubes by retro-orbital sinus puncture, from 14-h fasted hamsters anesthetized with CO_2. On day 27, the hamsters were killed by decapitation under CO_2 anesthesia. Hearts and livers were excised and cleaned of blood with ice-cold saline, and the tissues were weighed. The hearts were fixed on corks for histological examination. Livers were freeze-clamped in liquid nitrogen and stored at −80°C until analyses. Aliquots of blood samples were allowed to clot at room temperature, centrifuged at 6000 × *g* for 10 min, and stored at 4°C until they were analyzed within two days for total cholesterol, total triglyceride, and high density lipoprotein cholesterol (HDL-C) content. The remaining serum was stored at −20°C for determination of serum apolipoprotein A-I, apolipoprotein B, alanine aminotransferase, and lipid peroxides at a later date.

Biochemical analysis. Serum measurements of cholesterol, triglyceride, and HDL-C were obtained by automated enzymatic methodology with the Abbott VP Super System (Abbott, Irving, TX) using Abbott enzymatic reagent kits (Abbott kit 6095 for cholesterol, 6097 for triglycerides, and 6039 for HDL-C). Serum alanine aminotransferase was measured *via* an enzymatic assay (Sigma kit 59 for aminotransferase; Sigma Chemical Co., St. Louis, MO) as an index of possible liver damage. Apolipoproteins A-I and B were measured using enzymatic assays (356 for apolipoprotein A-I and 357 for apolipoprotein B; Sigma Chemical Co.). The serum alanine aminotransferase, apolipoproteins A-I and B enzymatic assays were analyzed using the Abbott VP Super System. Serum and liver were thawed and analyzed for lipid hydroperoxides using an adaptation of a lipid hydroperoxide kit assay (Kamiya Biomedical, Thousand Oaks, CA) based on the method of Oshishi *et al.* (21). The lipid was extracted by the method of Folch *et al.* (22) and reconstituted in different quantities of isopropranol (different dilutions) due to the variable amounts of lipids in each sample. The absorbance of the sample was determined at 620 nm using a microplate reader (Titertek Multiskan Plus MKII; ICN Biomedicals, Cleveland,

OH) using a 620 nm filter. The concentration of TBARS in livers was assayed as described previously (18). Concurrent measurements of lipid hydroperoxides and TBARS were made, as previous research has indicated that concentrations of these indices appear to peak at different time points in hepatic tissues in relation to the induction of the oxidative stress (23). The cholesterol content of the dietary fats was determined by gas–liquid chromatography by the method of Heuck *et al.* (24). The fatty acid composition of the dietary fats was determined by gas–liquid chromatography following fatty acid methyl esters formation and has been described previously (25).

Histopathology. The heart was sectioned midway through the atria, and the aorta was cut distally to the sinus valves and fixed on a cork. Cryostat frozen sections, 10 μm thick, were cut at three different levels and stained with oil red O and counterstained with haematoxylin. The sections were examined microscopically, and a similar section of each aorta was scored from 0 to 10 according to a modification of the method of Roberts and Thompson (26). Aorta were examined for the presence of foam cells and lesions in the aortic sinus wall. The histology was done on 50% of hearts, taken *via* random allocation, in each diet group (i.e., six hearts per dietary treatment).

Statistical analysis. Statistical analysis of the data was performed using the Statistical Analysis System for personal computers (Version 6.04, 1992; SAS Institute, Cary, NC). Two-way analysis of variance was used to determine the influence of time and dietary treatment on parameters measured at the beginning and end of the experiment (body weight, triglycerides, cholesterol, HDL-C). In Experiment 1, for serum lipid measures, body weight change was used as a covariate in the statistical model of two-way analysis of variance (ANOVA). One-way ANOVA was used to determine whether there was a main effect of diet on cumulative food intake, liver weight, serum alanine transferase, apolipoproteins A-I and B, apolipoprotein A-I/B, serum liver lipid peroxides, and liver TBARS. In Experiments 1 and 2, when a significant difference occurred among the main effects of diet and time in two-way ANOVA analyses or when a significant main effect of diet was observed *via* one-way ANOVA, mean differences among treatment groups were identified by least significant means for *a priori* comparisons. In Experiment 1, the following *a priori* comparisons were done to determine the effect of dietary source of fat on serum lipoprotein and tissue lipid peroxidation concentrations: (i) BF vs. SAFF, (ii) SAFF vs. MHO(H-CHOL), and (iii) BF vs. MHO(H-CHOL). Pearson's correlations between serum lipids and apolipoproteins and serum measures of oxidative stress (lipid peroxides and TBARS) were performed. In Experiment 2, the specified comparisons were: (i) MHO(H-CHOL) vs. MHO(L-CHOL) + E, (ii) MHO(H-CHOL) vs. MHO(L-CHOL) + CHOL + E , and (iii) MHO(L-CHOL) + E vs. MHO(L-CHOL) + CHOL + E. Cumulative food intake in Experiment 2 was put as a covariate in the statistical model of two-way ANOVA for serum lipid measures. The values were expressed as means ± SE for all parameters measured. In all comparisons, $P < 0.05$ was considered as the minimum level of significance.

RESULTS

Experiment 1. Food intake, weight gain, and tissue weight. There were no significant differences in cumulative food intakes among the BF (170.9 ± 3.7 g), SAFF (163.0 ± 3.7 g), and MHO(H-CHOL) (155.1 ± 3.2 g) diets. Body weight change from day 0 to day 27 was significantly higher ($P < 0.05$) in the BF (+4.4 ± 2.1 g) and the SAFF (+5.3 ± 1.8 g) groups as compared to the MHO(H-CHOL) group (−1.9 ± 1.4 g). No differences in the magnitude of body weight change were found between the BF group and the SAFF group. Liver weight as a percentage of body weight, however, was lower ($P < 0.05$) in the BF (6.35 ± 0.11%) and the SAFF (6.08 ± 0.07%) groups as compared to fish oil-fed (6.70 ± 0.12%) group suggesting an association between fish-oil feeding and hepatomegaly. The BF and SAFF groups were not different from each other in terms of liver weight as a percentage body weight. Liver weights were not different among the dietary treatments (data not shown).

Serum cholesterol, triglycerides, and apolipoproteins. Because significant differences were observed in terms of body weight change, body weight change was included as a covariate in the two-way ANOVA statistical analyses for serum lipid analyses. Body weight as a covariate, however, did not statistically influence serum lipid concentrations. In general, for total serum cholesterol concentrations, significant main effects of diet ($P < 0.0001$), time ($P < 0.0001$), and a significant diet × time ($P < 0.0001$) interaction were observed (Table 2).

In terms of the changes in total serum cholesterol, a significant main effect of diet ($P < 0.0001$) was observed. As would be expected from the high-saturated fat content of BF, the BF group showed a significantly higher final concentration of serum cholesterol and a larger increase in total serum cholesterol than did hamsters fed the polyunsaturated SAFF diet (Table 2). The MHO(H-CHOL) hamsters had significantly higher final levels of serum cholesterol than did the hamsters fed either the SAFF and BF diets. In addition, hamsters fed the MHO(H-CHOL) diet had greater change in total serum cholesterol concentrations over the 27-d feeding period than the BF and the SAFF groups (Table 2), indicating a hypercholesterolemic action of MHO feeding relative to the other lipid-based diets.

For serum triglycerides measured on day 0 and day 27, significant main effects of diet ($P < 0.05$), time ($P < 0.005$), and a significant interaction of diet × time ($P < 0.05$) were observed. The change from day 0 to day 27 in serum triglyceride concentrations also demonstrated a significant main effect of diet ($P < 0.001$). The MHO(H-CHOL) group demonstrated higher final concentrations of triglycerides than either the BF or SAFF groups. The change (day 0–27) in triglyceride concentrations in the MHO(H-CHOL) hamsters was also significantly greater than in the BF- and SAFF-fed animals, indicating a hypertriglyceridemic effect of fish-oil intake. No differences were observed in either final serum triglyceride concentrations or change (day 0–27) in serum triglyceride concentrations between the BF and SAFF diets.

TABLE 2
Effects of Dietary Fats on Serum Concentrations of Total Cholesterol, Triglycerides, HDL-C, and Apolipoproteins A-I and B in Hamsters (Experiment 1)[a]

	BF	SAFF	MHO(H-CHOL)
Total cholesterol (mg/dL)			
Day 0	(12) 108.87 ± 5.73	(12) 97.29 ± 6.58	(12) 100.58 ± 4.49
Day 27	(12) 154.67 ± 7.54	(12) 101.62 ± 5.81[a]	(12) 183.86 ± 6.97[b,c]
Change	(12) 45.81 ± 8.81	(12) 4.33 ± 8.72[a]	(12) 83.28 ± 7.68[b,c]
Total triglycerides (mg/dL)			
Day 0	(12) 230.33 ± 10.19	(12) 205.80 ± 11.61	(12) 200.03 ± 14.15
Day 27	(12) 233.96 ± 21.35	(12) 222.35 ± 15.90	(12) 303.95 ± 26.65[b,c]
Change	(12) -6.37 ± 22.25	(12) 16.56 ± 10.36	(12) 103.92 ± 20.06[b,c]
HDL-C (mg/dL)			
Day 0	(10) 59.12 ± 2.33	(10) 54.52 ± 2.68	(10) 54.29 ± 2.00
Day 27	(12) 71.38 ± 3.86	(10) 76.17 ± 8.18	(12) 83.04 ± 8.80
Change	(10) 6.05 ± 2.65	(10) 6.62 ± 3.18	(10) 10.87 ± 5.52
Apo A-I (mg/dL)	(5) 64.50 ± 2.04	(5) 62.80 ± 1.53	(5) 61.50 ± 2.32
Apo B (mg/dL)	(12) 16.35 ± 1.57	(12) 15.21 ± 1.20	(12) 37.85 ± 2.33[b,c]
Apo A-I/Apo B	(5) 5.32 ± 0.43	(5) 5.10 ± 0.88	(5) 1.77 ± 0.17[b,c]

[a]Values are means ± SE. Number in parentheses equals number of hamsters. Hamsters were fed BF, SAFF, and MHO(H-CHOL)-based semipurified diets for 27 d. See Table 1 for other abbreviations. Means within each row with superscript letters indicate the following comparisons are significantly different ($P < 0.05$) by least significant means: [a]BF vs. SAFF; [b]SAFF vs. MHO(H-CHOL); [c]BF vs. MHO(H-CHOL). Body weight change (day 0–27) was used as a covariate in the statistical model of two-way analysis of varriance (ANOVA). Significance of main effects of two-way ANOVA for total serum cholesterol (diet: $P < 0.05$; time: $P < 0.005$; diet × time: $P < 0.05$), total serum triglycerides (diet, $P < 0.0001$; time: $P < 0.0001$; diet × time: $P < 0.0001$), serum high density lipoprotein-choleserol (HDL-C), (diet: nonsignificant; time: $P < 0.0001$; diet × time: nonsignificant). Significant main effects of one-way ANOVA for serum cholesterol change ($P < 0.0001$), serum triglyceride change ($P < 0.001$), serum apo B ($P < 0.0001$), serum apolipoprotein (Apo) A-I/Apo-B ($P < 0.001$).

ANOVA showed no significant diet or time effects on serum HDL-C concentrations. On the other hand, the MHO(H-CHOL) animals had concentrations of apolipoprotein B that were greater than hamsters fed the BF and SAFF diets. In addition, hamsters fed the MHO(H-CHOL) diet had significantly lower ratios of apolipoprotein A-I/apolipoprotein B than the other two diets.

Serum and liver lipid oxidation products. The source of fat influenced the levels of serum lipid peroxides as animals fed the MHO(H-CHOL) diet had significantly higher serum levels of lipid peroxides than the hamsters fed the SAFF and BF diets (Table 3). Liver lipid peroxide and TBARS levels were not affected by the source of fat. When all dietary treatments were grouped together, correlations were observed between serum lipid peroxides and a number of serum indices including total serum cholesterol (change) ($r = 0.42$; $P < 0.004$), total serum triglycerides (change) ($r = 0.47$; $P < 0.0009$), serum apolipoprotein B ($r = 0.69$; $P < 0.0001$), and the serum apolipoprotein A-I to apolipoprotein B ratio ($r = -0.72$; $P < 0.0004$).

Serum alanine aminotransferase and histopathology. There were no significant differences observed among the treatment groups in terms of serum alanine aminotransferase levels (data not shown). Morphometric evaluation of the histologic sections showed the absence of foam cells. No atherosclerotic lesions in the aortic sinus wall and no significant differences in intimal thickness were observed among dietary treatments (data not shown).

Experiment 2. Food intake, weight gain, and tissue weight. There were no differences in cumulative food intake between the MHO(H-CHOL) (161 ± 6 g) group and either the MHO(L-CHOL) + CHOL + E (148 ± 5 g) or the MHO(L-CHOL) + E (167 ± 5 g) groups; however, food intake was significantly lower ($P < 0.05$) in the MHO(L-CHOL) + CHOL + E group when compared with the MHO(L-CHOL) + E group. Although total weight gain did not differ among the dietary treatments (data not shown), liver weight at day 27 was significantly higher ($P < 0.05$) in the MHO(H-CHOL)-fed group (6.14 ± 0.37 g) as compared with the MHO(L-CHOL) + E (4.82 ± 0.21 g) and MHO(L-CHOL) + CHOL + E (5.14 ± 0.21 g) groups. Similarly, liver weight as a percentage of body weight was significantly higher in the MHO(H-CHOL)-fed animals (4.50 ± 0.20%) relative to the MHO(L-CHOL) + E (3.60 ± 0.07%) and MHO(L-CHOL) + CHOL + E (4.00 ± 0.20%) groups, indicating that the hepatomegaly associated with MHO feeding in Experiment 1 could be alleviated by high vitamin E intake. The MHO(L-CHOL) + E and MHO(L-CHOL) + CHOL + E diet groups were not significantly different from each other in terms of liver weight or liver weight as a percentage of body weight.

Serum cholesterol and triglycerides. Since significantly different amounts of diets were consumed, food intake was included as a covariate for the two-way ANOVA for assessing the statistical comparisons of serum lipid values in Experiment 2. However, differences in cumulative food intake did not contribute to significant changes in plasma lipid con-

TABLE 3
Effects of Dietary Fats on Serum and Liver Lipid Peroxides (LPO) and Liver Thiobarbituric Acid Reactive Substances (TBARS) in Hamsters (Experiment 1)[a]

	BF	SAFF	MHO(H-CHOL)
LPO			
Serum (nmol/mL)	(12) 13.56 ± 3.88	(12) 20.23 ± 5.90	(12) 62.00 ± 7.73[b,c]
Liver (nmol/mg fat)	(12) 2.50 ± 0.53	(12) 1.36 ± 0.23	(12) 2.11 ± 0.44
TBARS			
Liver (nmol/mg fat)	(12) 8.98 ± 2.02	(11 9.71 ± 3.38	(11) 10.78 ± 1.82

[a]Values are means ± SE. Number in parentheses equals number of hamsters. See Table 1 for other abbreviations. Hamsters were fed BF, SAFF, and MHO(H-CHOL)-based semipurified diets for 27 d. Means within each row with superscript letters indicate the following comparisons are significantly different ($P < 0.05$) by least significant means: [a]BF vs. SAFF; [b]SAFF vs. MHO(H-CHOL); [c]BF vs. MHO(H-CHOL). Significance of main effect of one-way ANOVA for serum LPO ($P < 0.0001$).

centrations. In general, for total serum cholesterol concentrations, significant diet ($P < 0.001$), time ($P < 0.01$), and diet × time ($P < 0.005$) interactions were observed (Table 4). A significant diet ($P < 0.05$) effect was observed for change (day 0–27) in serum cholesterol concentrations. For the MHO(H-CHOL) group, serum cholesterol increased significantly ($P < 0.05$) from baseline after 27 d of feeding. On the other hand, no significant differences were observed for the MHO(L-CHOL) + CHOL + E and the MHO(L-CHOL) + E groups with respect to the comparison of cholesterol concentrations on day 27 with baseline values. The MHO(H-CHOL)-fed hamsters also had significantly higher final concentrations of total serum cholesterol than did either the MHO(L-CHOL) + E or the MHO(L-CHOL) + CHOL + E groups (Table 4). Vitamin E, however, did not completely overcome the hypercholesterolemic effect of cholesterol as no significant differences were observed between the MHO(H-CHOL) and the MHO (L-CHOL) + E + CHOL groups when the change in serum cholesterol between day 0 and day 27 was measured. The change in serum cholesterol over the 27-d feeding period was significantly lower in the MHO(L-CHOL) + E animals as compared with the other groups (Table 4). Overall, these data suggest that the hypercholesterolemic action of MHO was partially inhibited by high vitamin E intake.

A significant diet ($P < 0.001$) effect was observed in terms of total serum triglycerides, and a significant diet ($P < 0.05$) effect was observed for change (day 0–27) in serum triglyceride concentrations. After the 27-d feeding, the serum

TABLE 4
Effects of Vitamin E and Cholesterol in MHO-Based Diets on Serum Concentrations of Total Cholesterol, Triglycerides, and HDL-C in Hamsters (Experiment 2)[a]

	MHO(H-CHOL)	MHO(L-CHOL) + CHOL + E	MHO(L-CHOL) + E
Total cholesterol (mg/dL)			
Day 0	(7) 150.00 ± 11.94[d]	(7) 125.52 ± 4.85	(7) 130.74 ± 9.49
Day 27	(6) 214.83 ± 32.48[a,b]	(7) 140.23 ± 25.54	(7) 85.74 ± 7.43
Change	(6) 64.83 ± 35.42[b]	(7) 27.48 ± 29.05[c]	(7) −51.53 ± 6.09
Total triglycerides (mg/dL)			
Day 0	(7) 227.32 ± 25.99	(7) 196.73 ± 12.92	(7) 181.69 ± 20.13
Day 27	(6) 332.03 ± 21.98[a,b]	(7) 230.75 ± 16.69[c]	(7) 165.76 ± 19.12
Change	(6) 156.72 ± 44.07[a,b]	(7) 34.02 ± 19.91	(7) −6.51 ± 22.90
HDL-C (mg/dL)			
Day 0	(7) 103.63 ± 7.77[d]	(7) 83.81 ± 4.51[d]	(7) 87.90 ± 10.70[d]
Day 27	(6) 65.47 ± 6.04[a,b]	(7) 50.69 ± 3.17	(7) 49.32 ± 2.56
Change	(6) −38.16 ± 10.00	(7) −31.18 ± .63	(7) −41.01 ± 8.60

[a]Values are means ± SE. Number in parentheses equals number of hamsters. See Tables 1 and 2 for abbreviations. Hamsters were fed for 27 d one of three semipurified diets which consisted of: (i) a high-cholesterol MHO(H-CHOL); (ii) a low-cholesterol MHO which also contained high amounts of vitamin E (2.5 mg vitamin E/g oil) equivalent to a dietary concentration of 375 mg/kg [MHO(L-CHOL) + E]; and (iii) the low-cholesterol MHO(L-CHOL) + E oil (2.5 mg vitamin E/g oil) which was supplemented with United States Pharmacopeia cholesterol (595 mg/kg diet) [MHO(L-CHOL) + CHOL + E] to match the high-cholesterol content found in MHO(H-CHOL). Means within each row with superscript letters indicate the following comparisons are significantly different ($P < 0.05$) by least significant Means: [a]MHO(H-CHOL) vs. MHO(L-CHOL) + CHOL + E; [b] MHO(H-CHOL) vs. MHO(L-CHOL) + E; [c]MHO(L-CHOL) + E vs. MHO(L-CHOL) + CHOL + E. Means within each column with the supercript letter [d]indicate that the comparison of day 0 vs. day 27 is significantly different ($P < 0.05$) by least significant means. Cumulative food intake (day 0–27) was used as a covariate in the statistical model of two-way ANOVA. Significance of main effects of two-way ANOVA for total serum cholesterol (diet: $P < 0.001$; time: $P < 0.01$; diet × time: $P < 0.005$), total serum triglycerides (diet, $P < 0.001$; time, nonsignificant; diet × time: nonsignificant), serum HDL-C (diet: $P < 0.0001$; time: $P < 0.0001$; diet × time: $P < 0.0001$). Significance of main effect of one-way ANOVA for serum cholesterol change ($P < 0.05$) and serum triglyceride change ($P < 0.05$).

triglyceride concentrations were higher in the MHO(H-CHOL)-fed animals relative to the other diet groups, suggesting that the hypertriglyceridemia produced by MHO feeding was inhibited by the supplemental vitamin E. In addition, a hypertriglyceridemic effect of dietary cholesterol occurred as total serum triglyceride concentrations were significantly higher in the MHO(L-CHOL) + CHOL + E group as compared with the MHO(L-CHOL) + E animals. Also, when the change between initial and final triglyceride concentrations was compared, the MHO(H-CHOL) group had a significantly greater change in triglyceride concentrations as compared with both the MHO(L-CHOL) + E and the MHO(L-CHOL) + CHOL + E groups.

For serum HDL-C, significant diet ($P < 0.0001$), time ($P < 0.0001$), and diet × time interaction ($P < 0.0001$) effects were observed. All three diet groups showed a significant ($P < 0.0001$) drop in serum HDL-C values from baseline after the 27-d feeding. Serum HDL-C at day 27 was significantly higher in the MHO(H-CHOL) group than in either the MHO(L-CHOL) + E group or the MHO(L-CHOL) + CHOL + E group, but no significant differences were observed among all treatments when changes in HDL-C concentrations were analyzed.

Liver lipid peroxides. Livers from hamsters fed the MHO(H-CHOL) diet had significantly higher ($P < 0.05$) lipid peroxide concentrations (0.29 ± 0.05 nM/mg protein) as compared with either the MHO(L-CHOL) + E (0.15 ± 0.02 nM/mg protein) group or the MHO(L-CHOL) + CHOL + E (0.15 ± 0.03 nM/mg protein) group supporting the idea that supplemental vitamin E produced an inhibitory effect on MHO-mediated hepatic lipid peroxidation. The MHO(L-CHOL) + E animals did not differ significantly in terms of liver peroxide concentrations from the MHO(L-CHOL) + CHOL + E hamsters.

DISCUSSION

A possible etiological role of oxidative stress in hyperlipidemia has been suggested from animal studies showing that a deficiency of vitamin E, an important antioxidant that protects cell membranes against oxidative damage and lipid peroxidation, increases tissue cholesterol (5). The administration of fish oils to animals has previously been indicated to cause a low body content of vitamin E due to enhanced *in vivo* lipid peroxidation of n-3 fatty acids (27), and supplementation of vitamin E well above normal physiological requirements was needed in both mice (28) and primates (29) to normalize tissue vitamin E concentrations. Moreover, induction of oxidative stress in rats by administration of xenobiotic compounds, such as phenobarbital, has been demonstrated to increase total cholesterol and LDL-cholesterol (30). Conversely, supplementation of vitamin E at a level of 2000 mg vitamin E/kg diet has been shown to decrease plasma cholesterol concentrations in the Watanabe heritable hyperlipidemic rabbit (31). The findings of the present study lend further support for the concept that oxidative stress could play a significant role in disrupting cholesterol metabolism which can be ameliorated *via* high intakes of vitamin E.

Our findings show that the hyperlipidemic response in the hamster to a high intake of fish oil is related to the oxidative challenge induced by this diet. Experiment 1 showed that intake of MHO was associated with increased serum lipid peroxidation relative to hamsters fed either BF or SAFF-based diets. This observation is consistent with findings of higher serum concentrations of lipid peroxidation products observed in humans (13) and experimental animals such as rabbits (10) and rats (12) following fish oil supplementation. It is well established that polyunsaturated fats increase the requirement for vitamin E by increasing the susceptibility of cell membranes to oxidative degradation (32). As observed previously with n-6 PUFA diets (14), levels of tissue lipid peroxidation products were not increased by high n-6 fatty acid intake as compared with the high saturated fatty acid intake *via* the BF diet.

The high concentrations of serum lipid peroxides in the MHO(H-CHOL)-fed animals suggest that the body content of vitamin E was not adequate to protect highly oxidizable n-3 PUFA from oxygen-free radical attack. In support of this concept, the fish oil diets with a high vitamin E content in Experiment 2 were associated with lowered tissue lipid peroxidation. In the second experiment, fish oils with a high tocopherol content were associated with a serum cholesterol-lowering action even when matched for the higher cholesterol content of the hyperlipidemic MHO. The highly significant correlations between serum levels of lipid peroxidation and various apolipoprotein and lipoprotein fractions also suggest that the increased levels of oxidative stress could contribute to the adverse MHO(H-CHOL)-induced apolipoprotein and lipoprotein profiles. The results thus present the interesting possibility that depletion of tissue vitamin E and lipid peroxidation could be involved in the hyperlipidemic response of the hamster to fish oils. Human studies support the concept that supplemental vitamin E could affect the serum lipid-lowering effects of fish oil as subjects receiving vitamin E demonstrated a more marked fish oil-induced decrease in blood triglyceride concentrations than those receiving fish oil alone (13). As blood cholesterol concentrations were unaffected by vitamin E supplementation in the presence of fish oil intake, Haglund *et al.* (13) suggested that triglyceride synthesis might be more disrupted by peroxidation of fatty acids than cholesterol synthesis. The present study does not support this hypothesis in the hamster as both blood components were adversely affected by the MHO(H-CHOL) treatment. Moreover, both the cholesterol and triglyceride serum fractions showed lower concentrations (Table 4) in the presence of high intake of vitamin E when fish oil was given in Experiment 2. Differences in fish oil metabolism between the human and the hamster could account for the differential results observed in terms of serum cholesterol concentrations between the two studies.

In spite of increases in lipid peroxidation induced by the fish oil supplement, lipid peroxidation did not exert athero-

genic effects over the 27-d study period. This result does not discount the possiblity that atherogenic lesions may have occurred at a later period in the feeding study as aortic lesions were visible in fish oil-fed rabbits after five months of feeding (11). Although fatty streaks and foam cells have previously been reported in the Golden Syrian hamster after a four-week feeding (33), the atherogenic effects were caused by feeding 15% BF in the presence of much higher levels of cholesterol (3%) than was used in the present study (0.088%).

A major confounding effect between the two types of MHO oils was the higher cholesterol content of the MHO(H-CHOL). Despite the addition of matching levels of cholesterol to the MHO(L-CHOL), however, significant differences existed in terms of concentrations of serum cholesterol and triglycerides between the two oils. Moreover, only the MHO(H-CHOL) group experienced a significant rise in serum cholesterol concentrations from baseline values (Table 4). In Experiment 2, the serum lipid concentrations of MHO(L-CHOL) + CHOL + E were intermediary between those of the other two groups, thereby indicating that the high cholesterol content of MHO(H-CHOL) was partially responsible for the hyperlipidemic effect of the MHO(H-CHOL) oil. The minimal differences in fatty acid content between the MHO(H-CHOL) and the MHO(L-CHOL) oils were not likely responsible for the observed differences in serum lipid concentrations from the feeding of these two oils. Data from animal feeding studies that have compared fish oils with different fatty acid compositions with dietary levels of cholesterol adjusted to be the same. These studies have shown that the dietary ratios of 20:5n-3 to 20:4n-6 (16) and 20:5n-3 to 22:6n-3 (17) were the variables that caused differences in the hypolipidemic action among fish oils from different species. In particular, a hypolipidemic action was associated with fish oils with more than threefold higher dietary 20:5n-3/20:4n-6 ratios in contrast to fish oils that showed no hypolipidemic effect (16). In the present study, however, the differences in these dietary ratios were small between the two oils (Table 1). In fact, the trends in these ratios observed in the two MHO, deviated in the opposite direction. Hence, in terms of hypolipidemic action the hyperlipidemic MHO without added vitamin E had a higher 20:5n-3/20:4n-6 ratio (25.4) as compared to the hypolipidemic MHO with a high vitamin E content which had a ratio of 17. Four- to tenfold differences in the 20:5n-3 to 22:6n-3 ratio among fish oils have been indicated to result in differential decreases in plasma concentrations of total cholesterol, LDL, and HDL (17). The dietary 20:5n-3 to 22:6n-3 ratio, however, was identical in the two MHO, 1.3. These data support the claim that difference in the vitamin E content of the oils rather than the fatty acid composition was the major factor involved in the observed differences in lipid-lowering action between the two oils.

An increase in the serum triglyceride concentrations and a decrease in the apolipoprotein A-I/apolipoprotein B ratio have all been previously observed in the Golden Syrian hamster following feeding of a fish oil-based semipurified diet (8). In contrast to the present experiments, however, Hayes *et al.* (8) did not observe the hypercholesterolemic response to fish-oil feeding. The contrasting results in terms of serum cholesterol observed between our two studies could be accounted by differences in strain of Syrian hamster. Although the source of Syrian hamster was not indicated in the Hayes *et al.* study, a variability has been noted among Syrian hamsters from different commercial sources with respect to their lipidemic responses to dietary parameters (34).

Fish-oil intake in our study resulted in an increase in the liver weight/body weight ratio as in other studies (35). However, unlike other work (36), no increase in serum alanine aminotransferase was observed with fish-oil intake. Some investigators have suggested that the hepatomegaly could be related to the peroxisomal proliferation induced by fish-oil feeding and that the peroxisomal proliferation was a cause of the reduced synthesis and secretion of very low density lipoprotein triglyceride (37). Although peroxisomal proliferation was not measured, the fish oil-induced hepatomegaly in both Experiments 1 and 2 was associated with serum triglyceride-raising rather than triglyceride-lowering effects of fish oil, suggesting that the hepatomegaly may be dissociated from triglyceride-lowering effects of fish oil. As the hepatomegaly and hepatic lipid peroxidation were decreased in the presence of vitamin E supplementation, it is possible that fish oil-induced hepatomegaly could be related to oxidative stress at the hepatic level.

The present study shows that dietary vitamin E can modulate fish oil-induced changes in blood lipid metabolism and that a diet containing high amounts of fish oil induces an oxidative challenge. Lipid peroxidation and tocopherol depletion could be involved in the hyperlipidemic response of the hamster to fish oils since high vitamin E intake was associated with the production of hypolipidemic effects when MHO was fed. The highly significant correlations between serum levels of lipid peroxidation and various apolipoprotein and lipoprotein fractions lend further support to a role of lipid peroxidation in the diet-induced alterations in serum lipoprotein metabolism. Additional studies are needed in the hamster to investigate the extent to which fish oil consumption increase the requirement for antioxidant nutrients. Overall, the present studies indicate that the relationship between diet-induced oxidative stress and hyperlipidemia has been largely overlooked and is deserving of further study to fully evaluate its importance.

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A Diet Containing Myristoleic Plus Palmitoleic Acids Elevates Plasma Cholesterol in Young Growing Swine

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ABSTRACT: The objective of this study was to test the effect of a novel fatty acid mixture, enriched with myristoleic and palmitoleic acids, on plasma lipoprotein cholesterol concentrations. Weanling pigs were assigned to one of six groups and each group received a diet differing in fatty acid composition. Diets were fed for 35 days and contained 10 g added cornstarch/100 g (to provide baseline data) or 10 g added fatty acids/100 g. For those diets containing added fatty acids, extracted lipids contained 36% myristoleic plus palmitoleic acid combined (14:1/16:1 diet), 52% palmitic acid (16:0 diet), 51% stearic acid (18:0 diet), 47% oleic acid (18:1 diet), or 38% linoleic acid (18:2 diet). With the exception of the cornstarch diet, all diets contained approximately 30% myristic acid. There were no significant differences in weight gain across treatment groups ($P = 0.22$). All diets caused a significant increase in triglycerides and in total, low density lipoprotein, high density lipoprotein, and very low density lipoprotein cholesterol. The increase in total plasma cholesterol from pretreatment values was greatest in pigs fed the 14:1/16:1 and 18:1 diets. However, the increase in low density lipoprotein cholesterol from the pretreatment concentration was greatest in the 14:1/16:1-fed pigs. Increases in very low density lipoprotein cholesterol above pretreatment concentrations were lowest in 16:0-fed pigs and greatest in 18:1-fed pigs. Dietary fatty acids elicited changes in plasma fatty acids which generally were reflective of the diets, although the 18:0 diet did not alter plasma fatty acid concentrations and the 16:0 diet increased plasma 16:0 only at the end of the study. These results demonstrated that the combination of myristoleic plus palmitoleic acids increased plasma cholesterol in young pigs, suggesting that fatty acid chain length, rather than degree of unsaturation, is primarily responsible for the effects of fatty acids on circulating lipoprotein cholesterol concentrations. *Lipids 31*, 849–858 (1996).

In the United States, coronary heart disease is a leading cause of debilitating illness and death. Among several factors contributing to the increase in coronary heart disease, plasma lipid and lipoprotein cholesterol levels are the most highly correlated. The usefulness of these biochemical measures in predicting risk has been demonstrated by the findings of such epidemiological studies as the Framingham Heart Study (1). Dietary recommendations for reducing the risk of heart disease include those by the National Institutes of Health (2) and the National Cholesterol Education Program (3). Generally, the recommendations are for dietary fat to be limited to 30% of total energy or less and for saturated fatty acid intake to be limited to 10% or less of total energy.

Recommendations to reduce saturated fatty acid intake are based on research or epidemiological investigations comparing diets high in saturated, monounsaturated, or polyunsaturated fats (4). Typically, diets high in saturated fatty acids are enriched with lauric (12:0), myristic (14:0), or palmitic acid (16:0), whereas diets high in unsaturated fatty acids contain elevated concentrations of oleic (18:1), linoleic (18:2), or linolenic acid (18:3) (5,6). Thus, previous studies that addressed the effect of fatty acid saturation generally did not concurrently control fatty acid chain length.

More recent investigations have addressed the effects of fatty acid chain length (7–9). In nonhuman primates (7), a diet containing lauric plus myristic acids elicited greater concentrations of total and low density lipoprotein (LDL) cholesterol, and a similar effect was observed in humans fed diets enriched with either a synthetic high-lauric acid oil or palm oil (8). However, an investigation with piglets fed infant formulas (9) indicated that fatty acid positional distribution confounds the effects of fatty acid chain length on plasma cholesterol concentrations.

To our knowledge, no previous investigation has compared the effects of both chain length and degree of unsaturation on lipoprotein cholesterol concentrations. The current study takes advantage of the availability of a fatty acid mixture containing myristoleic acid combined with palmitoleic acid to compare the effects of shorter monounsaturated fatty acids (the myristoleic/palmitoleic acid mixture) to the more common 18-carbon oleic acid. Within this investigation, the effects of these monounsaturated fatty acids on lipoprotein cholesterol concentrations also were compared to the effects of the saturated fatty acids, palmitic and stearic acid, and the polyunsaturated fatty acid, linoleic acid.

MATERIALS AND METHODS

Animals and treatments. Pigs were obtained from sows housed at the Texas A&M University Department of Animal Science. Procedures for the handling and sampling of animals

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Abbreviations: apo, Apolipoprotein; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

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TABLE 1
Baseline Diet Composition[a]

	Diet	
	Cornstarch	Fatty acid
Composition (g/100 g as fed)		
Sorghum	56.48	56.48
Soybean meal	29.00	29.00
Lysine HCl, 98.8%	0.14	0.14
Cornstarch	10.00	—
Fatty acids	—	10.00
Dicalcium phosphate	1.56	1.56
Limestone	0.92	0.92
Vitamin mix[b]	0.50	0.50
Trace mineralized salts[c]	0.25	0.25
Salt	0.25	0.25
Medication[d]	1.00	1.00
Calculated content (%)		
Crude protein	18.00	18.00
Total lipids	2.50	12.50
Lysine	1.05	1.05
Calcium	0.80	0.80
Phosphorus	0.65	0.65
Energy density (kcal/100 g as fed)	391	441

[a]Diet exceeds all National Research Council requirements.
[b]Composition of the vitamin mix; retinyl palmitate, 2.3 mg/kg; cholecalciferol, 6.4 μg/kg; all-*rac*-α-tocopheryl acetate, 44 mg/kg; menadione sodium bisulfate, 2.8 mg/kg; riboflavin, 7.7 mg/kg; *d*-pantothenic acid, 30 mg/kg; niacin, 33 mg/kg; choline, 287 mg/kg; and cyanocobalamin, 44 μg/kg.
[c]Contributed the following mg per kg of diet: Cu, 10; Fe, 100; I, 0.3; Mn, 15; Zn, 100; Se, 0.3.
[d]Provided 55 mg carbodox/kg of diet.

were approved by the Texas A&M University Animal Care Committee.

Each treatment group contained seven pigs. The pigs were weaned at about 28 d of age. Weanling pigs were fed a sorghum-based diet containing neither added fat nor cornstarch for one week postweaning (Table 1) and then were allotted to one of six treatments groups. One of six littermates from each of seven sows was assigned to each diet group. Five groups received test diets containing 10 g added fatty acids/100 g (by wet weight); one group received the soybean meal diet containing corn starch substituted by weight for the added fatty acids. The cornstarch-fed group was included to provide baseline data, and to allow for the evaluation of production effects that were independent of dietary fatty acids.

Fatty acids or corn starch were mixed with the other dietary components weekly in a food mixer. The pigs were allowed free access to the food, and fresh water was available at all times. Diets were fed for 35 d, and pigs were weighed weekly.

The added fatty acids contained approximately 45% myristoleic plus palmitoleic acid combined, 95% palmitic, 95% stearic, 78% oleic, or 63% linoleic acid, as determined by fatty acid analysis in our laboratory (Table 2). Because the myristoleic/palmitoleic acid mixture also contained approximately 33% myristic acid, myristic acid (Sigma Chemical Co., St. Louis, MO) was added to all diets containing added fatty acids. The fatty acids were obtained from the Henkel Corporation (Cincinnati, OH). The myristoleic/palmitoleic

TABLE 2
Fatty Acid Composition of the Diets

	Diet group					
Fatty acid	Cornstarch	14:1/16:1	16:0	18:0	18:1	18:2
Composition of fatty acid mixtures, g total identifiable fatty acids/100 g[a]						
14:0	—[b]	33.4	0.5	nd[c]	1.8	0.1
14:1	—	30.2	0.1	nd	1.4	nd
16:0	—	5.4	94.7	4.0	3.8	3.3
16:1	—	14.6	0.2	nd	6.7	0.2
18:0	—	nd	4.4	94.8	0.2	0.8
18:1	—	13.3	nd	nd	78.4	24.9
18:2n-6	—	0.7	nd	nd	5.2	63.4
18:3n-3	—	0.1	nd	nd	0.2	7.2
Composition of diets, g total lipid/100 g diet[d]						
14:0	0.21	25.8	31.1	28.8	28.2	31.3
14:1	0.03	23.8	nd	nd	0.78	nd
16:0	14.2	7.7	51.6	6.0	5.7	6.0
16:1	0.54	11.9	nd	nd	3.9	0.03
18:0	2.5	0.48	3.2	50.9	0.69	0.87
18:1	27.1	0.65	4.2	4.8	46.7	19.0
18:2n-6	50.0	17.5	7.3	8.9	13.1	37.6
18:3n-3	6.1	12.1	0.05	0.51	1.6	5.3

[a]Composition of fatty acid mixtures as received.
[b]Measurement not applicable.
[c]nd = Not detectable.
[d]Fatty acid composition of the total lipids of the diets (as fed). The baseline diet (with no added fat) was calculated to contain 2.5% lipids, whereas the other diets contained 12.5% (2.5% endogenous lipids plus 10% added lipids). Myristic acid was added to the 16:0, 18:0, 18:1, and 18:2 diets to equal the amount of 14:0 observed in the 14:1/16:1 diet (approximately 33%).

acid mixture (provided without charge) was Emery 889, a nonfood-grade fatty acid mixture. Palmitic, stearic, oleic, and linoleic acids were Emersol food grade fatty acids, 6343, 6353, 6321, and 315, respectively.

After mixing the fatty acids with the basal diet, the fatty acid composition of each diet was as indicated in Table 2. The enrichment of the diets containing added myristic acid was apparent. The cornstarch diet contained a high percentage of oleic and linoleic acids; however, because the cornstarch diet contained only 2.5% total lipid, the actual amounts of oleic and linoleic acid were less than 0.6 and 1.1 g/100 g diet, respectively. In contrast, the oleic and linoleic acid diets contained 5.7 g oleic acid/100 g diet and 4.6 g linoleic acid/100 g diet, respectively (calculated from the data in Tables 1 and 2). Although the fatty acid mixtures used to enrich the diets were not pure, only the primary fatty acids (and the added myristic acid) contributed significantly to the fatty acid composition of each diet.

Plasma sample collection. Blood samples were obtained by vena caval puncture 7 d prior to the feeding trial and at weeks 1, 3, and 5. This schedule allowed two-week intervals between sampling times. The animals were allowed access to food prior to the first sample and were deprived of food approximately 16 h prior to obtaining the subsequent blood samples. Samples were collected and added to tubes containing EDTA (50 g/L final concentration), and then centrifuged at 2,500 × *g* for 15 min to isolate the plasma.

Plasma analyses. Total cholesterol was quantified with Sigma Diagnostics cholesterol reagent, which measures cholesterol enzymatically and is a modification of the method of Allain *et al.* (10). Lipoprotein cholesterol concentration was measured utilizing the LipoPhor System (Quantimetrix, Hawthorne, CA). By this system, lipoproteins were separated according to their molecular size and shape by discontinuous gel electrophoresis on polyacrylamide gels. The plasma sample was applied to the polyacrylamide gel tube with a loading gel solution containing Sudan Black B, which binds proportionally to the relative amount of cholesterol in each lipoprotein fraction. After electrophoresis was completed, the percentage of each of the separated lipoproteins was quantified with a Beckman DU-7 spectrophotometer with scanning and integrating software (Beckman Instruments, Irvine, CA). The relative amounts of very low density lipoprotein (VLDL), high density lipoprotein (HDL), and LDL cholesterol were calculated by factoring the percentage of each fraction by the total cholesterol of the plasma sample. Scans of control human plasma supplied by the manufacturer and from the pigs indicated nearly identical rates of migration of each lipoprotein cholesterol fraction (data not shown). In the human plasma samples, LDL cholesterol accounted for 52% of the total cholesterol, whereas in the porcine sample LDL cholesterol accounted for less than 40% of the total cholesterol, and HDL cholesterol was the predominant cholesterol fraction.

Quantitative triglyceride determination of 10 μL plasma utilized a modified procedure by Bucolo and David (11) and employed a triglyceride reagent (Sigma Chemical Co.). Briefly, triglycerides were hydrolyzed by microbial lipoprotein lipase. The liberated glycerol was converted to glycerol-3-phosphate by glycerol kinase and was subsequently oxidized to dihydroxyacetone phosphate by glycerol-3-phosphate dehydrogenase. The resulting NADH + H^+ reduces 2-(*p*-iodophenyl)-3-*p*-nitrophenyl-5-phenyltetrazolium chloride to formazan in the presence of microbial diaphorase. Formazan has an absorbance at 500 nm, and the reaction is linear for the range of triglycerides observed in plasma.

Fatty acid analysis. Fatty acid composition of 5 mL plasma from weeks 1 and 5 was measured by gas–liquid chromatography. Samples of the dietary fats (100 mg) and the actual diets (5 g) also were analyzed for fatty acid composition. The diet samples were obtained weekly, pooled within treatment, and subsampled for fat extraction. Lipid extraction was according to Folch *et al.* (12), and the extracted lipids were methylated by the Morrison and Smith (13) procedure, except that boron triflouride was increased to 7.2 g/sample. Subsequently, the fatty acid methyl esters were analyzed using a Packard Chrompack gas chromatograph (Model 437a; Chrompack Inc., Claritan, NJ) with a stainless steel column (3 mm × 10 m) packed with Chromasorb W-Aw 80/100. Injector and detector temperatures were 225 and 215°C, respectively. Oven temperature was 180°C initially. After 8 min (following the appearance of methyl palmitoleate), the oven temperature was increased by 10°C/min to a final temperature of 210°C. The flow rate of the carrier gas, nitrogen, was 22 mL/min. To each sample, 5 mg of an internal standard (methyl laurate) were added immediately prior to injection. A mixture of 20 mg each of trimyristin, tripalmitin, tristearin, and triarachidin was extracted, saponified, and methylated to calculate lipid recovery during the extraction process (approximately 80%). Another standard of known composition was run to verify the identity of the fatty acids in the samples. Fatty acid peaks determined by gas chromatography were then used to calculate the amount of fatty acids (mmol/L) according to the calculations described by Slover and Lanza (14).

Statistical analyses. For animal weights, differences from the initial values (7 d prior to the feeding trial) were calculated, and the data were analyzed using the general linear models procedure of the Statistical Analysis System (15). The model included diet, time, diet × time, and animal within diet.

Plasma measurements also were analyzed by the general linear models procedure (14) in two manners. Plasma values for pigs fed added fatty acids were compared to respective values from the cornstarch-fed group. Also, mean differences from initial (pretreatment) values over the course of the study were calculated within each treatment group, and the mean differences were compared across treatment groups. The model for both analyses included diet, time, diet × time, and animal within diet. Means were separated with Scheffe's multiple-comparisons test using a predetermined significance of $P < 0.05$ for all comparisons.

RESULTS

Animal growth. There were no significant differences across treatment groups in weight gain ($P = 0.22$) (Fig. 1A); average daily gains generally increased throughout the study (data not shown in tabular format). Because feed intake was based on pen averages, it was not possible to analyze this measure statistically. Average daily intake was low initially, and increased progressively until week 4 (Fig. 1B). The low rate of gain and intake during the first week of the study may have been caused by the young age at which these animals received dietary treatments (one week postweaning). Aversion to the diets was not apparent because intakes were similar in all treatment groups, particularly during the first four weeks of the study. By week 4, average daily intake appeared to decline in several groups, including the cornstarch-fed group (Fig. 1B). This experiment terminated on July 31, and the apparent decline in average daily intake may have been in response to the elevated ambient temperature.

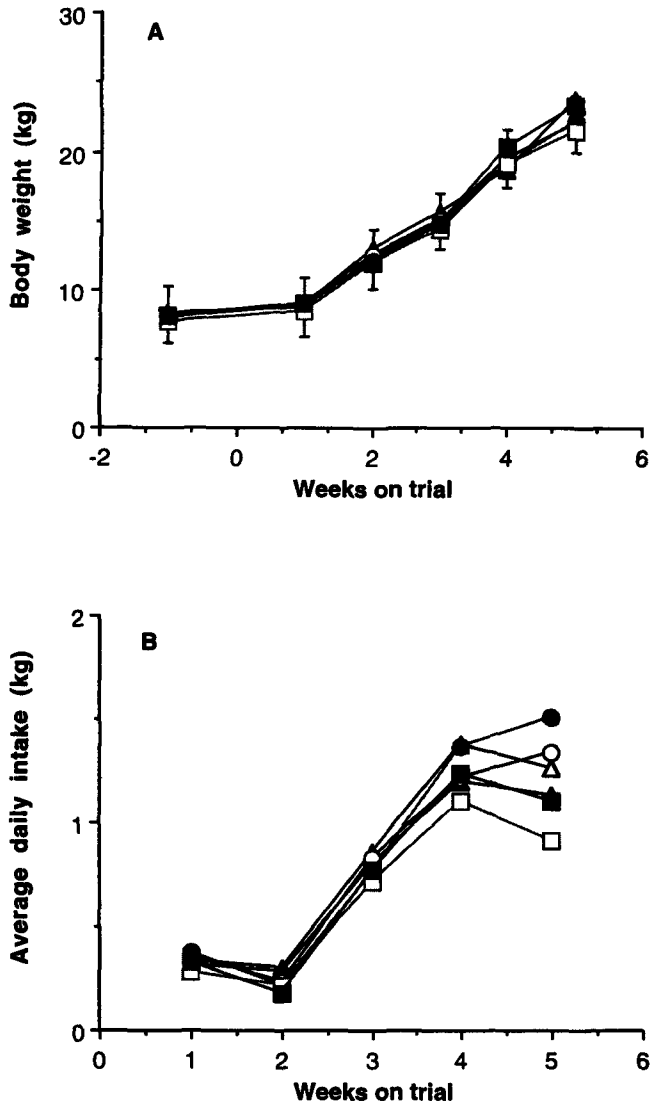


FIG. 1. Body weights and average daily feed intake of pigs fed diets varying in specific fatty acids. A, Body weight; B, average daily intake. Each data point represents a pen average for seven animals per pen. △, Cornstarch diet; ▲, 14:1/16:1 diet; ○, 16:0 diet; ●, 18:0 diet; □, 18:1 diet; ■, 18:2 diet.

Plasma cholesterol fractions and triglycerides. Plasma cholesterol concentrations were comparable to those reported by others for piglets and growing pigs (9,16,17), and HDL cholesterol constituted approximately 50% of total cholesterol (Fig. 2). In the cornstarch-fed pigs, average total cholesterol (2.78 mmol/L) was approximately one-half the value observed in humans (6,8,18) and nonhuman primates (5,7).

After one week of treatment, total cholesterol had risen in all groups, including the cornstarch-fed group (Fig. 2A). There was a signficant diet × time interaction for total ($P = 0.003$), LDL ($P = 0.006$), and HDL ($P = 0.04$) cholesterol concentrations, indicating that the changes with time varied among treatment groups. The pigs receiving the added myristoleic plus palmitoleic acid had the highest plasma total cholesterol throughout the study. At weeks 1 and 3, plasma cholesterol also was significantly elevated in pigs fed the added oleic acid, and at week 3, total cholesterol was elevated in pigs fed the linoleic acid diet. Total cholesterol declined markedly between weeks 3 and 5; this same pattern was observed in all cholesterol fractions (Fig. 2).

The LDL cholesterol concentrations for all groups were the same initially and followed the same general pattern as total cholesterol throughout the study (Fig. 2B). Throughout the study, LDL cholesterol concentrations were higher in pigs fed the myristoleic/palmitoleic acid diet than in the cornstarch-fed pigs. Diets containing oleic acid (weeks 1 and 3) and linoleic acid (week 3) also caused elevated LDL cholesterol concentrations (Fig. 2B). An identical pattern was observed for HDL cholesterol (Fig. 2C). There was not a significant diet × interaction for plasma concentrations of VLDL cholesterol (Fig. 2D). Lipoprotein cholesterol concentrations in pigs fed the palmitic or stearic acid-enriched diets were not affected by the inclusion of fatty acids in the diets. Plasma triglycerides also were unaffected by dietary fatty acids (Fig. 2E).

Overall means for differences from initial (pretreatment) concentrations for each of the cholesterol fractions (pooled across time) are listed in Table 3. There was a significant diet effect for total, LDL, and VLDL cholesterol. Total and LDL cholesterol differences from initial values were greatest in pigs fed the myristoleic/palmitoleic acid diet, whereas VLDL cholesterol was greatest in the oleic acid-fed pigs. Pigs fed stearic acid exhibited the lowest plasma total, LDL, and HDL cholesterol concentrations.

Plasma fatty acid composition. The groups receiving fatty acid-enriched diets exhibited markedly higher plasma myristic acid relative to the cornstarch-fed group (Fig. 3A), reflecting the addition of myristic acid to these diets. Plasma myristoleic (Fig. 3B) and palmitoleic acid (Fig. 3D) increased ($P <$ 0.0005) in pigs fed the myristoleic plus palmitoleic acid-en-

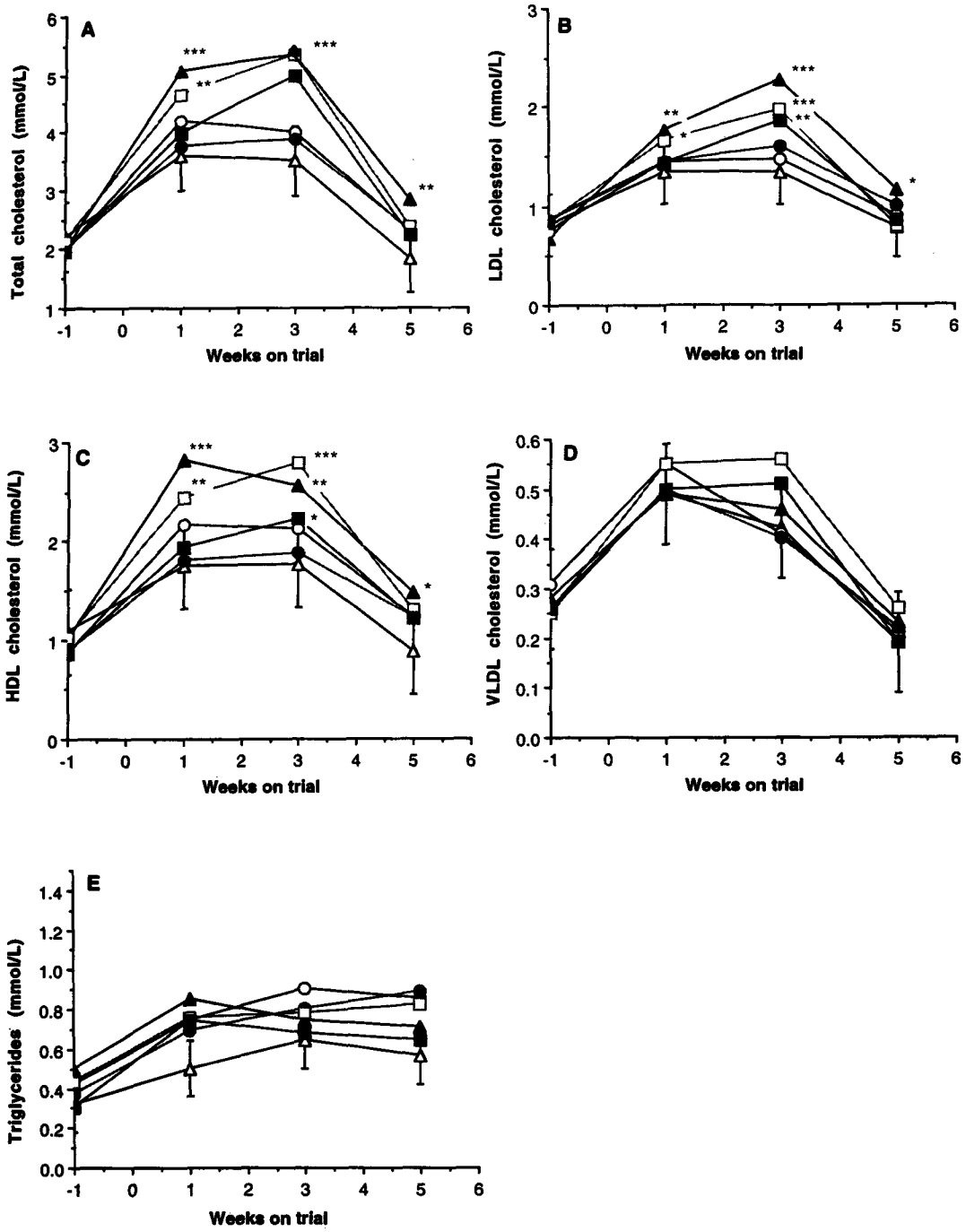


FIG. 2. Plasma lipoprotein cholesterol and triglyceride concentrations in pigs fed diets varying in specific fatty acids. Each data point is the mean for seven animals per treatment group. The vertical bars affixed to the points for the cornstarch-fed group represent the pooled standard deviation. A, Total cholesterol; B, low density lipoprotein (LDL) cholesterol; C, high density lipoprotein (HDL) cholesterol; D, very low density lipoprotein (VLDL) cholesterol; E, triglycerides. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$. Each data point represents a pen average for seven animals per pen. △, Cornstarch diet; ▲, 14:1/16:1 diet; ○, 16:0 diet; ●, 18:0 diet; □, 18:1 diet; ■, 18:2 diet.

riched diet. By week 5, pigs fed added palmitic acid exhibited elevated plasma palmitic acid (Fig. 3C).

Plasma stearic acid increased with time in all groups, but was unaffected by diet (Fig. 4A). None of the treatment groups exhibited greater plasma oleic acid than the cornstarch-fed group (Fig. 4B). By week 5, plasma linoleic acid was elevated in all treatment groups except those fed added stearic acid, relative to the cornstarch-fed group (Fig. 4C). In pigs fed the linoleic acid diet, plasma linoleic acid was elevated relative to the cornstarch-fed group by week 1. Neither

TABLE 3
Overall Means for Differences from Initial Values for Plasma Lipid Concentrations (mmol/L) in Pigs Fed Diets Varying in Specific Fatty Acids[a]

	Diet group						
Value	14:1 plus 16:1	16:0	18:0	18:1	18:2	Pooled (SD)	Significant effects
Total cholesterol	1.83[b]	1.16[c]	1.00[c]	1.49[bc]	1.31[c]	0.59	D, T
LDL cholesterol	0.78[b]	0.39[c]	0.36[c]	0.48[c]	0.39[c]	0.31	D, T
HDL cholesterol	0.96	0.74	0.57	0.85	0.69	0.43	T
VLDL cholesterol	0.12[bc]	0.10[c]	0.12[bc]	0.21[b]	0.15[bc]	0.11	D, T
Triglycerides	0.03	0.04	0.04	0.04	0.04	0.03	T

[a]Data are least square means for each set of seven pigs, pooled across all measurement time periods. Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein; D, significant diet effect ($P < 0.05$); T, significant time effect; D × T, significant diet × time interaction, as determined by analysis of variance. When analysis of variance indicated a significant diet effect, means were separated by Scheffe's multiple comparison test. Means in a row without a common superscript are different ($P < 0.05$).

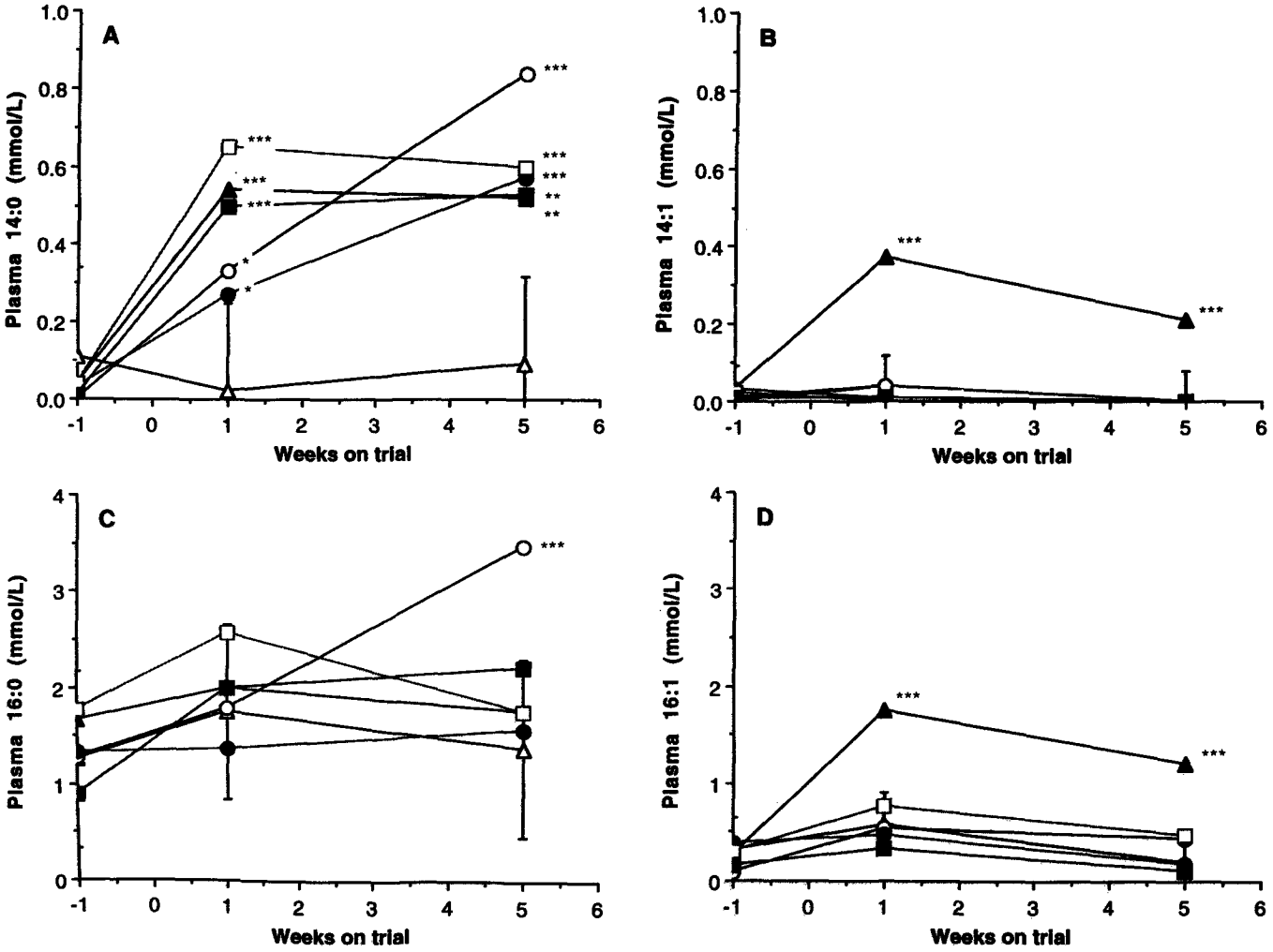


FIG. 3. Plasma 14- and 16-carbon fatty acid concentrations in pigs fed diets varying in specific fatty acids. Each data point is the mean for seven animals per treatment group. The vertical bars affixed to the points for the cornstarch-fed group represent the pooled standard deviation. A, Plasma myristic acid; B, plasma myristoleic acid; C, plasma palmitic acid; D, plasma palmitoleic acid. $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$. Each data point represents a pen average for seven animals per pen. △, Cornstarch diet; ▲, 14:1/16:1 diet; ○, 16:0 diet; ●, 18:0 diet; □, 18:1 diet; ■, 18:2 diet.

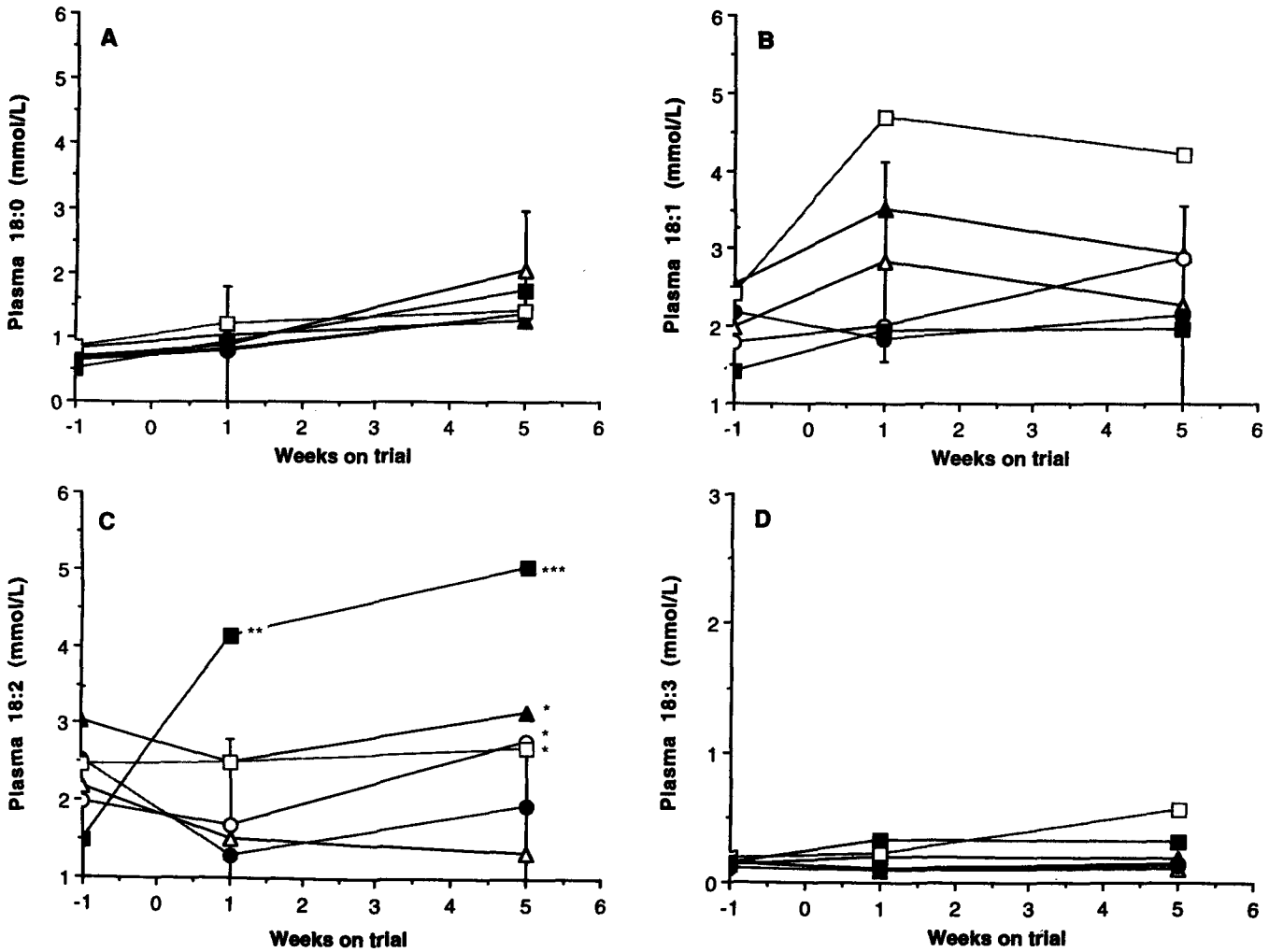


FIG. 4. Plasma 18-carbon fatty acid concentrations in pigs fed diets varying in specific fatty acids. Each data point is the mean for seven animals per treatment group. The vertical bars affixed to the points for the cornstarch-fed group represent the pooled standard deviation. A, Plasma stearic acid; B, plasma oleic acid; C, plasma linoleic acid; D, plasma linolenic acid. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$. Each data point represents a pen average for seven animals per pen. △, Cornstarch diet; ▲, 14:1/16:1 diet; ○, 16:0 diet; ●, 18:0 diet; □, 18:1 diet; ■, 18:2 diet.

plasma linolenic (Fig. 4D) nor arachidonic acid (data not shown) was affected by dietary treatment or time.

Overall means for differences from initial (pretreatment) concentrations for each of the plasma fatty acids (pooled across time) are listed in Table 4. Plasma myristic acid increased an average of approximately 0.25 mmol/L in those pigs fed diets containing added fatty acids; this represented a fivefold increase from initial values in plasma myristic acid. The marked increases in plasma myristoleic and palmitoleic acid (Figs. 3B and 3D) in pigs fed the myristoleic plus palmitoleic acid-enriched diet were evident in the mean changes from initial values (Table 4). It is unusual in that the palmitic acid and linoleic acid-enriched diets caused similar mean increases in plasma palmitic acid concentrations (approximately 0.8 mmol/L).

As indicated in Figure 4A, plasma stearic acid increased with time, but there was neither a diet effect ($P = 0.76$) nor a diet × time interaction ($P = 0.96$). Plasma oleic acid increased with time in several of the treatment groups (Fig. 3B), so the diet × time interaction was not significant ($P = 0.50$). However, the greatest mean increase from initial concentrations in plasma oleic acid was elicited by the oleic acid-enriched diet (Table 4). The largest increase from initial values for any plasma fatty acid was observed for plasma linoleic acid (2 mmol/L) in pigs fed diets enriched with this fatty acid. In the stearic acid-fed pigs, the plasma linoleic acid concentrations declined with time. Plasma linolenic and arachidonic acids were affected by neither diet nor time (Table 4).

DISCUSSION

Studies of the cardiovascular system of swine and the characteristics of their lipoproteins have established the pig as a model for the investigation of metabolic diseases in humans. High-fat and/or high-cholesterol diets in swine induce atherosclerosis (19,20). Swine, like humans, have a genetically di-

TABLE 4
Overall Means for Differences from Initial Values for Plasma Fatty Acid Concentrations (mmol/L) in Pigs Fed Diets Varying in Specific Fatty Acids[a]

	Diet group						
Value	14:1 plus 16:1	16:0	18:0	18:1	18:2	Pooled (SD)	Significant effects
Myrisitic acid	0.26	0.31	0.21	0.32	0.27	0.23	D, T, D × T
Myrisitoleic acid	0.17[b]	0.01[c]	−0.02[c]	−0.01[c]	0[c]	0.08	D, T, D × T
Palmitic acid	0.15[c]	0.88[b]	0.08[c]	0.26[c]	0.80[b]	0.91	D, T, D × T
Palmitoleic acid	0.83[b]	0.21[c]	−0.04[c]	0.22[c]	0.05[c]	0.32	D, T, D × T
Stearic acid	0.24	0.29	0.29	0.31	0.56	0.90	T
Oleic acid	0.46[c]	0.42[c]	−0.12[c]	1.33[b]	0.34[c]	1.29	D, T
Linoleic acid	0.14[c]	0.16[c]	−0.60[d]	0.08[c]	2.00[b]	1.29	D, D × T
Linolenic acid	0.04	−0.01	0	0.13	0.11	0.02	NS
Arachidonic acid	−0.14	0.11	−0.06	−0.24	0.50	0.03	NS

[a]Data are least square means for each set of seven pigs, pooled across all measurement time periods. Abbreviations as in Table 3. When analysis of variance indicated a significant diet effect, means were separated by Scheffe's multiple comparison test. Means in a row without a common superscript are different ($P < 0.05$).

verse propensity within their species to develop atherosclerosis, which has made possible many investigations of the interaction between nutrition and genetic background in the etiology of cardiovascular disease (21). One limitation of swine is that HDL cholesterol constitutes 50% or more of their total cholesterol (Fig. 2; Ref. 22). However, because both humans and swine have similar anatomy of the coronary arterial system and similar LDL receptors on liver membranes, and because both display a high correlation between aortic cholesterol and serum LDL-cholesterol levels (21), swine are considered useful models for the study of human cardiovascular disease. With this in mind, this investigation sought to document the effects of a diet enriched with 14- and 16-carbon monounsaturated fatty acids on lipoprotein cholesterol concentrations in young swine. The ultimate goal was to clarify the relative importance of fatty acid chain length and unsaturation in determining the effects of fatty acids on serum cholesterol in humans.

The marked elevation, and subsequent decline by week 5, in cholesterol fractions in all groups (including the cornstarch-fed group) was unexpected. In a previous study (22), mature pigs were fed diets containing high oleic acid (80%) sunflower oil or beef tallow at a level of 10% of the diet by weight. Plasma total cholesterol rose during the first four weeks (from 1.4 to 2.7 mmol/L in both groups). However, after eight weeks, plasma cholesterol had declined only marginally in the mature pigs (to 2.4 mmol/L), unlike the large decline observed in the young pigs of the present study. Food intake declined in all but the stearic acid-fed group during the last week of this investigation, which may have been responsible for the reduction in plasma cholesterol concentrations.

In spite of an apparent lack of a steady state during the course of this investigation, there was a consistent response of LDL cholesterol to the diet containing myristoleic plus palmitoleic acids. Reiser (23) asserted that the hypercholesterolemic effect of fatty acids was in part due to relative absorption of fatty acids, rather than degree of unsaturation. Correspondingly, plasma concentrations of myristoleic and palmitoleic acids were elevated markedly in pigs fed the diet enriched with these fatty acids. These data are insufficient to indicate relative absorption of the various dietary fatty acids. However, adding myristoleic and palmitoleic acids to the diet profoundly increased the availability of those fatty acids in plasma.

Previous research demonstrated that oils containing myristic acid increased plasma total cholesterol (24,25). The inclusion of myristic acid in the fatty acid-enriched diets increased plasma myristic acid to over 0.5 mmol/L by the end of the current study, which may have contributed to the hypercholesterolemic effects of the myristoleic/palmitoleic acid diet.

One primary objective of this investigation was to compare the effects of an 18-carbon monounsaturated fatty acid (i.e., oleic acid) to the effects of shorter chain monounsaturated fatty acids on cholesterol concentrations. In humans, enriching diets with oleic acid results in lower total cholesterol than diets enriched with saturated fatty acids (17,26–28); the same has been observed in nonhuman primates (5). Oleic acid-enriched diets do not decrease LDL cholesterol in rats; nor do they affect HDL cholesterol (29). In the current investigation, the oleic acid-enriched diet increased total cholesterol from pretreatment values nearly to the extent of the myristoleic/ palmitoleic acid-enriched diet. However, the primary effect of the oleic acid-enriched diet was to increase the concentration of VLDL (and to some extent HDL) cholesterol whereas, as stated previously, the myristoleic/palmitoleic acid diet increased both HDL and LDL cholesterol.

There are several mechanisms by which the myristoleic plus palmitoleic acid-enriched diet could have increased LDL cholesterol. Oleic acid stimulates rat hepatic cholesterol synthesis by activating hydroxy-methylglutaryl–CoA reductase (30), and other monounsaturated fatty acids may have the same effect. In guinea pigs, oleic acid slows LDL turnover by altering LDL composition (31), and the shorter chain monounsaturated fatty acids may have increased the concentration of LDL cholesterol by this mechanism.

Saturated fatty acids have been shown to block LDL cholesterol receptors (32,33), due primarily to impaired LDL re-

ceptor-mediated catabolism (34). It is unlikely that the myristoleic/palmitoleic-enriched diet worked *via* this mechanism, because neither added myrisitic acid nor palmitic acid increased LDL-cholesterol. Replacing dietary lauric and myristic acids with palmitic acid decreased total and LDL cholesterol in nonhuman primates, and concurrently decreased the apo B/apo A-I ratio (7). The myristoleic/palmitoleic-enriched diet may have increased total and LDL cholesterol in pigs *via* increased apo B production.

Another specific objective of this investigation was to demonstrate that 14- to 16-carbon fatty acids are hypercholesterolemic, whereas 18-carbon fatty acids are hypocholesterolemic, regardless of the degree of unsaturation. However, cholesterol concentrations in pigs fed diets enriched with either palmitic or stearic acid were not different; nor were they different from cholesterol concentrations in pigs fed linoleic acid.

The design of this experiment did not allow statistical comparison of food intake; however, average daily intake by week 5 was greatest in pigs fed diets enriched with stearic acid. Thus, feeding saturated fatty acids, particularly stearic acid, as free fatty acids may have resulted in poor absorption. Although Bonanome and Grundy (26) provided evidence for absorption of dietary stearic acid in humans, Ockner *et al.* (35) demonstrated that dietary saturated fatty acids, once released from triacylglycerols, are absorbed more slowly than unsaturated fatty acids. This has been confirmed by several recent investigations. Monsma and May (36), Renaud *et al.* (37), and Brink *et al.* (38) indicated that stearic acid in the *sn*-1,3 positions of dietary triacylglycerols is poorly absorbed, particularly in the presence of elevated dietary calcium (38). A recent study in piglets (9) indicated that this also applies to palmitic acid in the *sn*-1,3 position.

Our data suggest that dietary palmitic acid was absorbed more effectively than was dietary stearic acid: the plasma palmitic acid concentration (and increase from pretreatment levels) was greatest in pigs fed the palmitic acid-enriched diet; and, like all other dietary fatty acids except stearic acid, the palmitic acid diet increased HDL cholesterol. The lack of effect a significant effect of the stearic acid-enriched diet on any of the cholesterol fractions is taken as further evidence for poor absorption of dietary stearic acid in this study.

We also had predicted that the diet high in stearic acid would increase the plasma concentration of oleic acid. Bonanome and Grundy (39) suggested that absorbed stearic acid would be desaturated to form oleic acid. Stearoyl-CoA desaturase is active in porcine liver, adipose tissue, muscle, and intestinal mucosa (22), so the potential for desaturation exists at several porcine tissue sites. However, plasma oleic acid was not elevated in pigs fed the stearic acid-enriched diet in the present study; nor is dietary stearic acid readily interconverted to oleic acid in rat liver (36).

Unlike the diets containing saturated fatty acids, the linoleic acid-enriched diet elicited consistent and predictable results. The overall changes in total and LDL cholesterol were low, especially when compared to the increase elicited by the myristoleic/palmitoleic-enriched diet. Thus, for those diets enriched with unsaturated fatty acids, we observed three distinct responses: the myristoleic/palmitoleic acid diet caused marked elevations in LDL cholesterol; the oleic acid diet caused the greatest increase in VLDL cholesterol; and the linoleic acid diet increased neither. The singularly hypercholesterolemic nature of the myristoleic/palmitoleic acid diet leads us to conclude that fatty acid chain length, rather than degree of unsaturation, is responsible for the effects of dietary fatty acids on the plasma concentration of LDL cholesterol in young swine. Total and LDL cholesterol were much higher in pigs fed the myristoleic/palmitoleic diet and in those fed the 16-carbon, palmitic acid-enriched diet, which suggests that myristoleic acid was more hypercholesterolemic than the 16-carbon palmitoleic acid. Similar studies in humans await the availability of a food-grade source of myristoleic and palmitoleic acids.

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Fatty Acid Composition of Late Embryonic and Early Postnatal Rat Brain

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ABSTRACT: The fatty acid (FA) composition and distribution in a variety of phospholipids (PL) and neutral lipids (NL) at two discrete stages during the embryonic rat brain development were investigated. Over 96% of the FA were acylated into fetal brain PL at embryonic day 17 after the peak of neuronal proliferation and at embryonic day 20, one day prior to delivery. Phosphatidylcholine constituted approximately 60% of the total PL pool, phosphatidylethanolamine (PE) 30%, phosphatidylserine (PS) 6%, and phosphatidylinositol (PI) 4%. The diacylglycerols and triacylglycerols constituted 1–2% of the fetal brain lipids. α-Linolenic acid (18:3n-3) and linoleic acid (18:2n-6) were found in very low amounts in all fetal brain PL and NL. The percentage of the n-6 polyunsaturated FA, consisting of arachidonic acid (AA), 22:4n-6 and 22:5n-6, remained unchanged in all the fractions, except in PI, in which the proportion of AA increased. The concentration of docosahexaenoic acid (DHA) increased with age in all the fractions, with the bulk of accumulation accounted for by its increase in PE and, to a lesser extent, in PS. This finding suggests a "DHA accretion spurt" during the last three days of pregnancy.

Lipids 31, 859–865 (1996).

Docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6) are the major polyunsaturated fatty acids (PUFA) in the adult mammalian brain (1,2), and can be locally synthesized from their corresponding dietary precursors, α-linolenic acid (LNA, 18:3n-3) and linoleic acid (LA, 18:2n-6), respectively (3,4).

In a recent publication we have shown that the prenatal rat brain is also able to convert both LNA and LA into the higher long-chain polyunsaturated homologs (5). In the course of this study, it became apparent that only scant information exists on the fatty acid (FA) composition of the rat brain during the prenatal life. Sinclair and Crawford (6) studied the FA composition of the total brain in mid-term fetal and newborn rats, and Cunnane and Chen (7,8) performed a quantitative analysis of the FA composition of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and triacylglycerols (TG) in the postnatal, but not prenatal, rat brain. More recently, a detailed description of the FA composition of PC and PE in the prenatal guinea pig brain has been documented (9,10). There is practically no knowledge concerning the FA composition of the less abundant brain lipids, i.e., phosphatidylinositol (PI), phosphatidylserine (PS), or neutral lipids (NL), such as diacylglycerols (DG) and TG. Several of these quantitatively minor lipid fractions now have well established roles in cell function and signal transduction (11–13).

The present study was undertaken in order to provide detailed information on the FA composition of most abundant lipid fractions of the fetal rat brain at 17 and 20 d of gestation. The first time point selected represents the stage of cessation of neuronal proliferation, and the second time point reflects the FA status prior to delivery. The data obtained were compared to the FA composition of the lactating (postnatal day 8, P8) rat brain. The data herein suggest that during the late intrauterine life a major accumulation of brain DHA occurs, mainly in PE and PS lipids.

EXPERIMENTAL PROCEDURES

Animals and surgery. Pregnant Wistar (250–300 g body weight) rats (WIS Animal Breeding Center, Rehovot, Israel) were fed *ad libitum* chow containing 5 g lipid/100 g with the following FA composition: 13% 16:0, 4% 18:0, 24% 18:1, and 52% 18:2n-6, and 5% 18:3n-3. At days 17 and 20 of gestation (E17 and E20), dams were anesthetized by intramuscular injection of 50 mg ketamine (Parke Davis, Pontypool, Wales, United Kingdom) and 7 mg xylizine HCl (Bayer, Leverkusen, Germany) per kg body weight (0.2 mL/300 g body weight of a 1:1 mixture of 2% xylizine HCl-Rompun and 0.1 g/mL ketamine). An abdominal midline incision was performed, the two uterine horns were exposed, and the fetal brains were immediately placed in liquid nitrogen upon removal. The time elapsed between the incision of the fetal neck in preparation for brain removal (and thus causing fetal death), and the placement of the brain into liquid nitrogen was never more than 10 s. Similarly, approximately 200-mg portions of P8 rat brains also were removed.

Lipid extraction and analysis. After weighing, the brains were homogenized in 2 mL hexane/isopropanol (3:2, vol/vol) containing 5% butylated hydroxytoluene, using a Polytron ho-

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Abbreviations: AA, arachidonic acid; DG, diacylglycerol; DHA, docosahexaenoic acid; E17, E20, embryonic day 17, 20; FA, fatty acid; LA, linoleic acid; LNA, α-linolenic acid; NL, neutral lipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; P8, postnatal day 8; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; PUFA, polyunsaturated FA; TG, triacylglycerol.

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mogenizer (Kinematica, Luzern, Switzerland), according to Hara and Radin (14). A known amount of the internal standard heneicosanoic acid (21:0) was added to each sample before homogenization in order to quantitate the total brain FA content. The organic layer containing the lipid extract was separated from the residual tissue by low-speed centrifugation.

Nonpolar and polar lipids were separated by thin-layer chromatography on washed and preactivated silica gel G plates (Merck, Darmstadt, Germany). The solvent system used to separate DG and TG from free FA consisted of a freshly prepared mixture of hexane/ether/acetic acid (40:60:2.3, by vol) (15). Standard lipid mixtures (Sigma Chemical Co., St. Louis, MO) were run for identification of spots. The major phospholipids (PL)—PC, PE, PI, and PS—were separated by a mixture of chloroform/methanol/methylamine (40%) (130:70:30, by vol) (16). All the solvents were of high-performance liquid chromatographic grade.

Lipid spots resolved on thin-layer chromatography using the solvent mixture hexane/ether/acetic acid (40:60:2.3, by vol) were visualized under ultraviolet light after spraying with 0.2% dichlorofluorescein in ethanol. Individual spots were identified and scraped into glass tubes to which known amounts of heneicosanoic acid were added. Each sample was transmethylated, and total FA was quantitated based on the internal standard as described previously (17). The sum of TG, DG, free FA, and PL (the latter resided at the origin) accounted virtually for all the FA content present in the extract. The percentage weight of FA in each individual fraction was estimated, and the results are means ±1 SE in each individual fraction. Determinations were done on six E17 samples (each sample of three pooled brains), seven E20 samples (each sample of two pooled brains), and four P8 brains.

FA accretion rates were calculated as follows: for the intrauterine period up to E17, the brain FA content at E17 was divided by 17; for the next 3 d of fetal life, the difference between the FA content at E20 and E17 was divided by 3; finally, the difference between the FA content at P8 and E20 divided by 9 resulted in the accretion rate of the FA during the early postnatal period. Lipid phosphorus was determined according to Bartlett (18).

Statistical analysis. Analysis of the data was performed by the general linear model provided by the statistical analysis system for analysis of variance with repeated measures, nested design, where fetuses were nested within mothers. Specific differences between the developmental stages were determined by Tukey's procedure, with the α level set at 0.05. Values of various developmental periods designated by the same letters were not significantly different.

RESULTS

The changes in the brain weight and brain total FA content from E17 through E20 and up to P8 are depicted in Figure 1. Most of the increase in brain weight during this period was from day E20 to P8, but FA accumulation was maximal before birth.

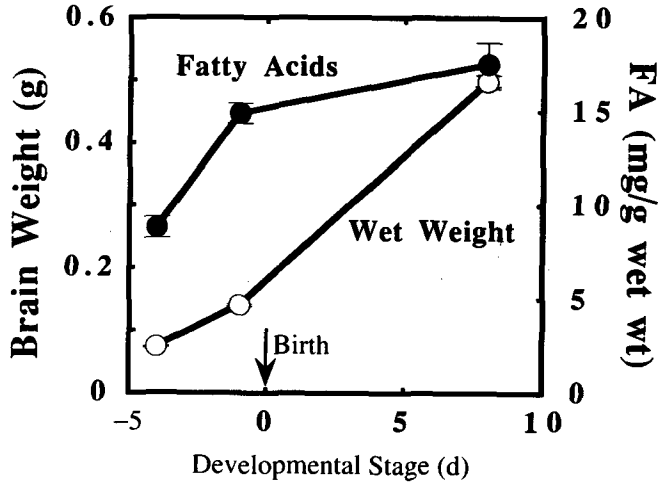


FIG. 1. Changes in the brain weight and total fatty acid (FA) content. Total brain weight (○) and brain FA concentration (●) during development. Values of brain weight are means ± SE of 69, 58, and 18 brains from embryonic day 17 (E17), embryonic day 20 (E20), and postnatal day 8 (P8) animals, respectively. FA concentrations are means ± SE of 12 E17 samples (each sample of three pooled brains), 10 E20 samples (each sample of two pooled brains), and 16 P8 brains.

The majority (96%) of the brain FA was associated with the PL pool (Table 1). The NL pool, consisting mainly of DG, TG, and free FA, accounted for the remaining 4% of the total brain FA. No differences were observed in the relative size of either pool during the developmental time points examined. Within the PL pool, PC and PE were the major fractions, constituting approximately 60 and 30% of total PL, respectively. PS and PI comprised approximately 6 and 4% of total PL, respectively.

The FA composition of the total brain lipids is shown in Table 2. The proportion of the saturated FA and the n-6 PUFA remained relatively constant. The percentage of the monounsaturated FA decreased by nearly 35% between E17 and P8. This decrease was accounted for by decreases in both 16:1 and 18:1. The n-3 PUFA almost doubled during this period, with most of the increase accounted for by changes in the amount of DHA.

The average daily accretion rates calculated for the major FA are depicted in Figure 2. On a semilog scale, DHA was

TABLE 1
Major Fatty Acids (FA) Containing Lipid Pools During Perinatal Stages of the Rat Brain[a]

Lipid	Weight percentage of FA		
	E17	E20	P8
Phospholipid	96.23 ± 0.87	96.16 ± 0.57	95.68 ± 0.47
Diacylglycerol	1.22 ± 0.36	0.87 ± 0.12	1.83 ± 0.10
Triacylglycerol	1.80 ± 0.14	1.91 ± 0.44	1.53 ± 0.50
Free FA	0.73 ± 0.46	1.06 ± 0.17	0.98 ± 0.09

[a]Results are means ± SE of the percentage of the fraction, determined by the weight of the FA in each as described in the Materials and Methods section. Determinations were done on six E17 (embryonic day 17) samples (each sample of three pooled brains), seven E20 (embryonic day 20) samples (each sample of two pooled brains), and four P8 (postnatal day 8) brains.

TABLE 2
Developmental Changes in FA Composition of Total Brain Lipids[a]

FA	Weight percentage			
	E17	E20	P8	*P*
14:0	1.96 ± 0.04[b]	1.99 ± 0.04[b]	1.65 ± 0.08[c]	0.03
16:0 DMA	2.45 ± 0.08[b]	2.10 ± 0.06[b,c]	1.85 ± 0.10[c]	0.02
16:0	28.36 ± 0.39	30.36 ± 0.68	28.51 ± 0.96	
16:1	5.01 ± 0.07[b]	4.64 ± 0.06[c]	3.15 ± 0.10[d]	0.0001
18:0 DMA	1.30 ± 0.12	1.37 ± 0.03	1.45 ± 0.12	
18:1n-9 DMA	0.61 ± 0.02[b]	0.44 ± 0.01[c]	0.30 ± 0.02[d]	0.0003
18:1n-7 DMA	0.50 ± 0.02[b]	0.64 ± 0.04[c]	0.17 ± 0.01[d]	0.0003
18:0	15.26 ± 0.23	15.40 ± 0.13	15.85 ± 0.58	
18:1	17.06 ± 0.18[b]	14.28 ± 0.05[c]	11.14 ± 0.17[d]	0.0001
18:2n-6	1.15 ± 0.03[b]	1.24 ± 0.04[b]	1.68 ± 0.08[c]	0.0018
18:3n-3	0.45 ± 0.06	0.25 ± 0.04	0.56 ± 0.14	
20:3n-6	0.41 ± 0.02[b]	0.49 ± 0.03[b]	0.59 ± 0.02[c]	0.0019
20:4n-6	13.98 ± 0.28[b]	12.39 ± 0.28[c]	14.52 ± 0.38[b]	0.0062
22:4n-6	3.38 ± 0.10[b,c]	2.90 ± 0.12[b]	3.59 ± 0.13[c]	0.036
22:5n-6	3.44 ± 0.09[b]	3.07 ± 0.12[b]	1.84 ± 0.07[c]	0.0001
22:5n-3	0.27 ± 0.03	0.28 ± 0.02	0.33 ± 0.02	
22:6n-3	6.43 ± 0.36[b]	8.55 ± 0.39[c]	12.82 ± 0.44[d]	0.0001
Σ Sat	45.59 ± 0.63	47.23 ± 0.77	45.00 ± 0.56	
Σ MUFA	22.07 ± 0.24[a]	19.28 ± 0.23[b]	14.30 ± 0.27[c]	0.0001
Σ n-6 PUFA	22.36 ± 0.40[a]	19.78 ± 0.32[b]	22.23 ± 0.51[a,b]	0.019
Σ n-3 PUFA	7.08 ± 0.36[a]	9.86 ± 0.35[b]	13.71 ± 0.41[c]	0.0001

[a]Results are mean weight % ± SE of FA from six E17 samples (each sample of three pooled brains), eight E20 samples (each sample of two pooled brains), and five P8 brains. DMA, dimethylacetal; Σ Sat, sum of saturated FA (14:0, 16:0, and 18:0); Σ MUFA, sum of monounsaturated FA (MUFA) (16:1 and 18:1); Σ n-6 PUFA, sum of the n-6 family of polyunsaturated FA (PUFA) (18:2, 20:3, 20:4, 22:4, and 22:5); Σ n-3 PUFA, sum of the n-3 family of PUFA (18:3, 22:5, and 22:6). Values not bearing the same superscript (b–d) are significantly different. Abbreviations as in Table 1.

the most pronounced PUFA to accumulate prior to birth. The average daily accumulation rate of DHA increased almost fivefold between E17 and E20 (from 39 μg/g/d to 187 μg/g/d), whereas that of AA increased only 1.3-fold (from 85 μg/g/d at E17 to 113 μg/g/d at E20). After birth, the daily acquisition rate of DHA decreased while that of AA remained constant.

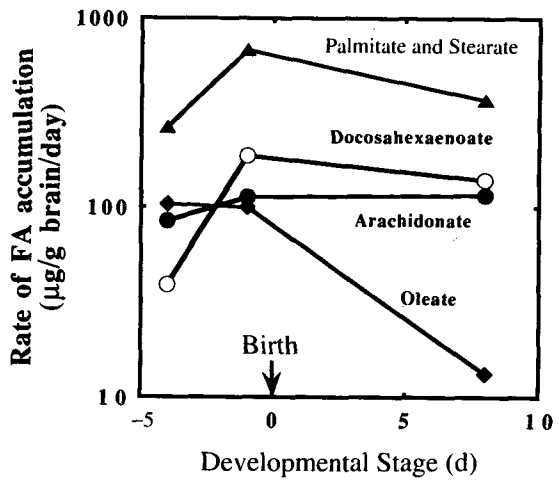


FIG. 2. Values are average daily accretion rates of the respective FA calculated for the following periods: up to E17 (day 4), between E17 and E20 (day 1), and between E20 and P8. DHA, docosahexaenoic acid; AA, arachidonic acid; OA, oleic acid; Sat., saturated acid. See Figure 1 for other abbreviation.

The FA composition of the major brain lipids is shown in Tables 3 and 4. The largest component of esterified FA was saturated. PE was the least saturated PL, mainly because this fraction included alk-1-enyl bonds (plasmalogen), which constituted approximately 40% of the total PE pool at E20 (Green, P., and Yavin, E., unpublished data). Although the total saturated FA were found in similar concentrations in most of the lipid fractions (Table 3), the types of saturated FA differed widely (Table 4). Thus, the major saturated FA in PC was 16:0, and in PI and PS it was 18:0. In PE, 16:0 and 18:0 were found in almost equal proportions. Most of the monounsaturated FA was comprised of 18:1 (Table 4), and was most abundant in PC (Table 4). The proportion of this FA group decreased in all the lipid fractions from E17 to P8, the most pronounced changes being observed in PS and PE.

The major long-chain PUFA were AA and DHA (Table 4). In PS and PE, 22:4n-6 and 22:5n-6 also were found in substantial proportions. At all the developmental points, PE contained most of the brain PUFA, both n-6 and n-3 forms (Table 3). Thus, although the percentage of n-3 PUFA was highest in PS, their absolute amount was severalfold greater in PE. Similarly, even though AA was the major PUFA in PI, amounting to 39.4–45.2% of the total FA in this fraction (Table 4), PE contained 3.4-, 3.8-, and 11.9-fold more AA on a weight basis than PI at E17, E20, and P8, respectively.

PS and PE were relatively enriched in DHA (Table 4) and contained most of the DHA esterified into the total brain PL (78.8, 84.2, and 91.5% at E17, E20, and P8, respectively, calculated from Figure 1, Table 4, and the relative amounts of the various PL). Moreover, most of the increase in total brain DHA observed during this period was accounted for by the increases of this FA in PE and PS. DHA also increased substantially in TG and DG (Table 4), but total esterified DHA in these NL fractions was less than in PC because of their small amount (Table 1).

Concomitantly with the increases in DHA described here, significant decreases in the proportion of 22:5n-6 were observed in all the lipid fractions (Table 4).

DISCUSSION

In this study we provide detailed information on the quantitative aspects of FA composition and accretion in the lipid fractions of the early developing rat brain. This information was deemed important because, in contrast to abundant data on the FA composition in the adult rodent brain (2,19–22), much less is known about the FA in the brain lipid fractions during fetal development (6,9,23). Furthermore, the changes of the FA in the various PL classes at these critical stages of the developing rat brain are still not complete. A detailed quantitative analysis of the PC and PE molecular species in the guinea pig fetal brain was recently reported (10), but data on the FA composition of PI and PS, as well as of the NL, were not provided. These latter lipids, although minor quantitatively, are

TABLE 3
Developmental Changes in FA Composition of PL and Neutral Lipids (NL) According to Degree of Saturation[a]

Lipid class	FA	Weight percentage E17	E20	P8	*P*
PI	Σ Sat.	44.28 ± 0.71[b]	41.80 ± 0.55[c]	43.70 ± 0.15[b]	0.003
	Σ MUFA	8.74 ± 0.87	8.25 ± 0.44	7.07 ± 0.28	
	Σ n-6 PUFA	44.84 ± 1.58	46.53 ± 0.97	46.22 ± 0.18	
	Σ n-3 PUFA	2.02 ± 0.22	2.81 ± 0.19	3.00 ± 0.26	
PS	Σ Sat.	48.00 ± 0.58[b]	45.46 ± 0.70[c]	49.60 ± 0.41[b]	0.002
	Σ MUFA	12.31 ± 0.77[b]	9.48 ± 0.19[c]	6.92 ± 0.20[d]	0.0001
	Σ n-6 PUFA	26.36 ± 0.62[b]	26.00 ± 0.56[b]	20.37 ± 0.28[c]	0.0001
	Σ n-3 PUFA	12.94 ± 0.83[b]	18.93 ± 0.61[c]	23.07 ± 0.36[d]	0.0001
PC	Σ Sat.	58.00 ± 0.45[b]	59.35 ± 0.28[b]	64.74 ± 0.48[c]	0.017
	Σ MUFA	32.59 ± 0.38[b]	28.08 ± 0.33[c]	24.58 ± 0.16[d]	0.012
	Σ n-6 PUFA	10.20 ± 0.31	10.12 ± 0.39	8.80 ± 0.33	
	Σ n-3 PUFA	1.95 ± 0.30	2.23 ± 0.07	1.45 ± 0.13	
PE	Σ Sat.	28.76 ± 0.17	29.40 ± 0.05	40.61 ± 0.82	
	Σ MUFA	11.91 ± 0.44[b]	10.47 ± 0.33[b]	8.23 ± 0.24[c]	0.02
	Σ n-6 PUFA	31.94 ± 0.34	29.00 ± 0.27	31.02 ± 0.44	
	Σ n-3 PUFA	11.70 ± 0.47	17.35 ± 0.20	19.33 ± 0.43	
DG	Σ Sat.	50.45 ± 0.99	46.50 ± 2.99	43.01 ± 1.70	
	Σ MUFA	17.24 ± 1.95	16.70 ± 2.27	11.00 ± 1.47	
	Σ n-6 PUFA	28.34 ± 1.58	27.35 ± 0.54	29.15 ± 1.13	
	Σ n-3 PUFA	4.63 ± 1.13	15.52 ± 3.10	16.83 ± 0.38	
TG	Σ Sat.	42.40 ± 1.10	44.30 ± 3.91	44.87 ± 1.30	
	Σ MUFA	27.05 ± 2.00	25.21 ± 3.21	21.93 ± 5.21	
	Σ n-6 PUFA	28.05 ± 1.21	25.51 ± 2.35	21.80 ± 2.37	
	Σ n-3 PUFA	3.38 ± 0.18[b]	8.41 ± 0.41[c]	11.80 ± 1.05[c]	0.009

[a]Abbreviations and statistics as in Tables 1 and 2. PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DG, diacylglycerol; TG, triacylglycerol; Sat., saturated.

essential for such cellular functions as enzyme activation (13) and second-messenger formation (11). For example, it has been demonstrated that PS is a necessary component in the activation of several protein kinase C isoforms (12). Moreover, it appears that the fatty acyl composition of PS is one of the regulatory factors in enzyme activation (24).

The bulk of weight increase of rat brain occurs postnatally, with the growth spurt paralleling myelinogenesis (25), whereas neuronal proliferation and differentiation are nearly complete by birth. Thus, neuronal membrane PL formation and FA accretion should be substantial at this period. Therefore, failure to acquire sufficient and appropriate PUFA at such critical periods in fetal development may have harmful and irreversible consequences for postnatal growth and function. In this study we show that FA accumulation is steeper than brain weight gain before birth; weight gain is more rapid while during the early postnatal period. The latter relationship between brain weight gain and FA accretion persists up to the fifth postnatal week (6). Thus, intrauterine life constitutes an important period of brain FA deposition.

The changes in the quantity of the long-chain PUFA are of special interest, as they, or their precursor FA, LA, and LNA, have to be obtained from the diet. In a previous developmental study, it has been shown that the bulk of the rat brain PUFA accumulates postnatally (6). Furthermore, in several species, during brain growth n-6 PUFA accumulation predated that of n-3 PUFA accumulation (26). In these reports, however, detailed analysis of fetal brain FA was not performed. The increase in the amount of the n-3 PUFA demonstrated in the present study was mainly due to the prominent accretion of DHA (Table 2). Actually, the strikingly accelerated rate of DHA accretion during the last three days of pregnancy (Fig. 2) constitutes a "DHA accretion spurt," analogous to the traditional concept of "brain growth spurt" coined by Dobbing (25). A similar period of prenatal rapid DHA accumulation was observed between day 25 and day 35 of gestation in the guinea pig (9). An increasing ratio of DHA to AA has been documented in the human fetal brain after 30 wk of gestation (27). However, in contrast to our results in the rat fetal brain (Table 2), the enhanced accumulation of DHA in the human fetal brain was accompanied by decreasing proportions of AA.

The sources of the long-chain PUFA in the fetal brain are mostly maternal (6), although it has been shown both *in vitro* (28) and *in vivo* (5) that the fetal rat brain has the ability to form long-chain PUFA from their precursors. The ability of

TABLE 4
Developmental Changes in FA Composition of Selected Brain Lipid Fractions[a]

Lipid	FA	Weight percentage			
		E17	E20	P8	*P*
PI	14:0	0.74	0.77 ± 0.16	0.14 ± 0.01	
	16:0	8.64 ± 1.48[b]	4.94 ± 0.34[c]	5.60 ± 0.18[c]	0.006
	18:0	35.27 ± 0.80[b]	36.67 ± 0.56[b,c]	37.95 ± 0.21[c]	0.05
	18:1	8.74 ± 0.87	7.93 ± 0.40	6.60 ± 0.29	
	18:2n-6	2.69 ± 0.58	0.56 ± 0.04	ND	
	18:3n-3	1.06	0.47 ± 0.13	0.62 ± 0.19	
	20:4n-6	39.41 ± 1.53[b]	42.42 ± 0.87[b]	45.16 ± 0.33[c]	0.009
	22:4n-6	1.21 ± 0.11[b]	1.41 ± 0.12[b]	0.73 ± 0.06[c]	0.007
	22:5n-6	1.53 ± 0.44[b]	1.53 ± 0.30[b]	0.44 ± 0.04[c]	0.01
	22:6n-3	1.49 ± 0.13[b]	2.34 ± 0.11[c]	2.38 ± 0.15[c]	0.03
PS	14:0	0.35 ± 0.05	0.62 ± 0.11	0.50 ± 0.04	
	16:0	9.07 ± 0.61[b]	6.50 ± 0.46[c]	8.12 ± 0.39[b,c]	0.02
	16:1	1.75 ± 0.15[b]	1.33 ± 0.03[c]	0.94 ± 0.05[d]	0.0003
	18:0	38.49 ± 0.23[b]	38.47 ± 0.35[b]	40.97 ± 0.15[c]	0.0001
	18:1	10.32 ± 0.62[b]	8.15 ± 0.17[c]	5.99 ± 0.16[d]	0.0001
	18:2n-6	1.38 ± 0.09[b]	0.67 ± 0.05[c]	0.28 ± 0.03[d]	0.0002
	18:3n-3	0.89 ± 0.27	0.28	0.41 ± 0.10	
	20:4n-6	9.67 ± 0.23	9.66 ± 0.63	9.48 ± 0.11	
	22:4n-6	7.31 ± 0.42[b,c]	7.24 ± 0.20[b]	6.17 ± 0.25[c]	0.04
	22:5n-6	7.50 ± 0.37[b]	7.73 ± 0.38[b]	3.97 ± 0.11[c]	0.0001
	22:5n-3	0.28 ± 0.02[b]	0.43 ± 0.01[c]	0.48 ± 0.03[c]	0.004
	22:6n-3	12.06 ± 0.79[b]	18.43 ± 0.60[c]	22.31 ± 0.28[d]	0.0001
PC	14:0	3.11 ± 0.09	3.34 ± 0.07	2.98 ± 0.14	
	16:0 DMA	0.17 ± 0.01	0.12 ± 0.01	ND	
	16:0	48.08 ± 0.46[b]	50.47 ± 0.16[b]	56.19 ± 0.49[c]	0.009
	16:1	8.88 ± 0.09[b]	7.32 ± 0.04[c]	6.52 ± 0.06[c]	0.009
	18:0 DMA	0.07 ± 0.005	0.10 ± 0.01	0.16 ± 0.01	
	18:1n-9 DMA	0.08 ± 0.01	ND	0.08 ± 0.01	
	18:0	6.81 ± 0.16[b]	5.55 ± 0.30[c]	5.75 ± 0.23[c]	0.06
	18:1	23.71 ± 0.32[b]	20.76 ± 0.30[c]	18.07 ± 0.16[d]	0.02
	18:2n-6	1.42 ± 0.03[b]	1.42 ± 0.06[b]	1.65 ± 0.08[c]	0.01
	18:3n-3	0.34 ± 0.09	ND	0.11 ± 0.02	0.04
	20:4n-6	6.98 ± 0.23	6.69 ± 0.29	6.07 ± 0.24	
	22:4n-6	0.78 ± 0.03[b]	0.84 ± 0.02[b]	0.41 ± 0.04[c]	0.03
	22:5n-6	0.73 ± 0.04[b]	0.71 ± 0.02[b]	0.20 ± 0.02[c]	0.02
	22:5n-3	0.05 ± 0.005	0.10 ± 0.01	0.07 ± 0.01	
	22:6n-3	1.50 ± 0.18	2.13 ± 0.07	1.23 ± 0.13	
PE	14:0	0.34 ± 0.01	0.37 ± 0.01	0.28 ± 0.03	
	16:0 DMA	8.14 ± 0.08[b]	6.96 ± 0.09[b]	0.76 ± 0.18[c]	0.01
	16:0	11.59 ± 0.13	11.21 ± 0.09	16.49 ± 0.57	
	16:1	1.48 ± 0.09	1.41 ± 0.10	0.87 ± 0.06	
	18:0 DMA	4.05 ± 0.20[b]	3.67 ± 0.70[b]	0.16 ± 0.04[b,c]	0.007
	18:1n-9 DMA	2.12 ± 0.12	1.37 ± 0.02	ND	
	18:1n-7 DMA	1.63 ± 0.08	1.78 ± 0.06	ND	
	18:0	16.83 ± 0.16[b]	17.83 ± 0.09[b]	23.85 ± 0.32[c]	0.06
	18:1	10.44 ± 0.34[b]	9.06 ± 0.24[b,c]	7.37 ± 0.26[c]	0.06
	18:2n-6	0.56 ± 0.03	0.73 ± 0.06	0.64 ± 0.14	
	18:3n-3	0.62 ± 0.12	0.30 ± 0.07	0.22 ± 0.07	
	20:4n-6	18.96 ± 0.18[b,c]	16.73 ± 0.29[b]	20.71 ± 0.32[c]	0.05
	22:4n-6	5.97 ± 0.06	5.38 ± 0.11	6.16 ± 0.19	
	22:5n-6	6.12 ± 0.09[b]	5.68 ± 0.10[b,c]	3.01 ± 0.07[c]	0.04
	22:5n-3	0.22 ± 0.002[b]	0.32 ± 0.003[b,c]	0.39 ± 0.02[c]	0.04
	22:6n-3	10.86 ± 0.36	16.72 ± 0.20	18.72 ± 0.38	
DG	16:0	27.01 ± 0.60	25.36 ± 1.89	23.50 ± 1.64	
	18:0	21.08 ± 1.18	18.49 ± 1.54	19.50 ± 0.29	
	18:1	13.50 ± 1.80	14.81 ± 1.71	10.99 ± 1.46	
	18:2n-6	1.54 ± 0.19	2.40 ± 0.36	1.27 ± 0.07	

(continued)

TABLE 4 (continued)

Lipid	FA	Weight percentage E17	E20	P8	*P*
DG	18:3n-3	2.81 ± 1.12	6.87 ± 1.42	4.92 ± 0.94	
	20:4n-6	17.84 ± 1.01	16.40 ± 1.54	23.38 ± 1.11	
	22:4n-6	2.26 ± 0.30	3.80 ± 0.55	2.72 ± 0.16	
	22:5n-6	6.45 ± 0.75	4.75 ± 1.01	1.80 ± 0.09	
	22:6n-3	2.52 ± 0.33	9.86 ± 1.76	11.92 ± 1.14	
TG	14:0	3.13 ± 0.11	3.68 ± 0.35	5.42 ± 0.87	
	16:0	29.04 ± 0.76	27.73 ± 2.54	25.07 ± 2.08	
	18:0	10.23 ± 0.23	12.54 ± 1.25	13.50 ± 1.80	
	18:1	21.68 ± 2.04	21.15 ± 3.34	21.92 ± 5.20	
	18:2n-6	4.70 ± 0.50	4.61 ± 0.87	6.53 ± 0.08	
	20:4n-6	11.16 ± 0.60	10.61 ± 0.77	15.27 ± 2.44	
	22:4n-6	4.01 ± 0.17	4.43 ± 0.47	ND	
	22:5n-6	5.73 ± 0.29	4.95 ± 0.66	ND	
	22:6n-3	2.93 ± 0.12[b]	7.65 ± 0.31[c]	11.82 ± 1.03[d]	0.004

[a]Abbreviations and statistics as in Tables 1 and 2. ND, not detected.

early postnatal rat brains to elongate-desaturate deuterium-labeled LA and LNA to their longer chain desaturated products has been demonstrated recently (29). Although the fetal liver does not supply long-chain PUFA to the fetal guinea pig brain (9), it does so in the postnatal developing mouse brain (30), the early postnatal rat (29), and in domestic felines (31).

The very low proportion of the precursor essential FA as compared to their long-chain products in the developing brain (Tables 2 and 4) suggests preferential uptake of the long-chain PUFA from the blood (32), local elongation-desaturation by the brain (5), or both. The low levels of brain LA and LNA were not due to dietary deficiency, because high proportion of these FA, especially of LA, were found in the 20-d fetal liver (Green, P., and Yavin, E., data not shown). The proportions of LA and LNA in the brain lipid do not change substantially during development, as adult rat (22) and mouse (33) brains also contain very low levels of these FA.

Provided adequate dietary conditions prevail, the FA composition of the individual brain PL in the adult rat and mouse is highly characteristic (2,19–21). In our study, this distinctive pattern of brain PL acyl chain composition was already evident (Table 4), implying that the mechanisms responsible for the final PL shaping (34) are present and active in the fetal brain. Some of them have been demonstrated in our previous study (5).

Two points of interest should be mentioned regarding the fetal accumulation of the PUFA in the brain PL between E17 and E20. First, the percentage of AA increased only in PI (Table 4), that of 22:4n-6 and 22:5n-6 remained almost unchanged in all the PL, but the concentration of DHA increased in all of them (Table 4). Second, the bulk of brain DHA accretion from E17 to E20 was accounted for by its increase mainly in PE and, to a lesser extent, in PS.

In the first postnatal week, a steep decrease in the proportion of 22:5n-6 was observed in all the fractions (Table 4). Because elevation of brain 22:5n-6 has been associated with dietary deficiency of n-3 FA (35), it may be postulated that during intrauterine life, a relative n-3 FA deficiency exists. Indeed, major increases in the proportion of DHA in most lipid fractions were observed between E17 and P8 (Table 4).

The FA composition of DG and TG was more variable than that of the PL (Table 4). The FA composition of the TG partially reflects that of their precursors, the DG (Table 4). It has been postulated by Cook (28) that the brain TG pool is important for the transfer of long-chain FA to PL. In the present study, a steep increase in the proportion of DHA occurred in TG (Table 4). In addition, even though the amounts of DG and TG in the fetal brain were negligible relative to the PL (Table 1), a very brisk turnover of labeled AA and DHA in these fractions was demonstrated (Green, P., and Yavin, E., unpublished data). Taken together, these data tend to confirm Cook's assumption (28).

In summary, substantial brain FA accumulation occurs during late gestation, with the accretion of DHA being most prominent. The bulk of DHA accumulation is accounted for by its increase in PE, and to a lesser amount, in PS. Because the fetus is totally dependent upon the maternal supply of nutrients for the acquisition of its FA (36), the "DHA accretion spurt" described in this study underscores the need for adequate maternal nutrition and an intact and functioning placental-fetal unit.

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The Effects of 3-Hydroxy-3-Methylglutaryl–CoA Reductase Inhibition on Tissue Levels of Carnitine and Carnitine Acyltransferase Activity in the Rabbit

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ABSTRACT: Recently, a new class of lipid lowering agents [3-hydroxy-3-methylglutaryl (HMG)–CoA reductase inhibitors] was introduced into clinical practice. The use of these agents could lead to a secondary deficiency in carnitine, which may manifest clinically as a myalgia/myositis—a side effect that is occasionally seen with this class of drugs. In the present study, we examined the effect of an HMG–CoA reductase inhibitor (lovastatin) on serum and tissue levels of carnitine and carnitine acyltransferase activities in the rabbit. Rabbits (n = 6) were fed chow containing lovastatin (30 mg/d) for 16 wk. Blood was collected and tissues (liver, heart, and skeletal muscle) harvested at sacrifice. Free and total carnitine were measured in serum and tissues by a radioenzymatic method. Carnitine acetyltransferase and carnitine palmitoyltransferase (CPT) activities were determined and expressed relative to DNA. Serum free (24.0 ± 2.6 vs. 29.4 ± 3.1 μM) and total (35.1 ± 4.7 vs. 52.8 ± 8.8 μM) carnitine levels increased significantly with 16 wk of treatment. This increase in total carnitine was mainly due to an increase in the levels of serum acylcarnitine (12.7 ± 3.1 vs 26.5 ± 5.7 μM). Tissue levels of total carnitine were significantly decreased by the treatment. Carnitine acetyltransferase was unaffected by the treatment, whereas there was a significant increase in the activity of CPT in the liver and heart.

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High concentrations of plasma low density lipoprotein cholesterol are believed to be a major risk factor in the development of atherosclerosis and coronary heart disease (1). With the advent of inhibitors of 3-hydroxy-3-methylglutaryl (HMG)–CoA reductase activity (the rate-limiting step in cholesterol biosynthesis), the treatment of hypercholesterolemia has improved considerably (2,3). Lovastatin is one of the more widely prescribed agents among the different HMG–CoA reductase inhibitors (2,3). Development of diffuse muscle pain and myositis, which occasionally progresses to frank rhabdomyolysis, is a rare side effect that can be seen with the use of these agents (4,5). Because these drugs inhibit the rate-limiting enzyme for cholesterol synthesis, their use would lead to an increase in the acyl-CoA/free CoA ratio within the liver cell. Under these circumstances, there may be a higher than normal carnitine requirement for the buffering of these acyl-CoA moieties through an esterification reaction yielding acylcarnitine and free CoA. Theoretically, under these circumstances, some patients may develop a secondary deficiency in muscle carnitine. Clinically, this may manifest as a myalgia/myositis.

Carnitine is found in virtually all cells of higher organisms. The primary function of this molecule is to transfer long-chain acyl-CoA across the inner mitochondrial membrane for β-oxidation (6). Carnitine may also function in other metabolic processes including the modulation of the acyl-CoA/CoASH ratio in mammalian cells (7). Ninety-eight percent of the total body carnitine store is in muscle; the remaining is present in extracellular fluid, liver, and other tissues existing in free and acylated forms. Carnitine acyltransferases (CAT) catalyze the formation of acylcarnitines from acyl-CoA. CAT and carnitine palmitoyltransferase (CPT) are two important enzymes involved in these reactions (8). Carnitine has been suggested to activate fatty acid oxidation and to prevent the accumulation of potentially toxic levels of cytosolic and mitochondrial acyl-CoA (9).

In this study, we examined the effect of 16 wk of administration of an HMG–CoA reductase inhibitor (lovastatin) on carnitine and acylcarnitine concentrations in chow-fed rabbits. In addition, we studied the effect of this drug on the activity of two carnitine related enzymes—CAT and CPT. Our results indicate that treatment of rabbits with an HMG–CoA reductase inhibitor for 16 wk alters their carnitine status.

MATERIALS AND METHODS

Animals. Female New Zealand white rabbits (2.0–2.5 kg) were used. They were divided into two groups: a control group (n = 6) fed Purina rabbit chow (Purina Mills, St. Louis, MO), and the experimental group (n = 6) fed a rabbit chow diet containing lovastatin (30 mg/d; Ralston Purina Canada Inc., Mississauga, Ontario, Canada) for 16 wk. Lovastatin was mixed with ethanol (2 mL) and rabbit chow (200 g). The control diet was prepared similarly without adding lovastatin.

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Abbreviations: CAT, carnitine acetyltransferase; CPT, carnitine palmitoyltransferase; HMG, 3-hydroxy-3-methylglutaryl.

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The animals were housed in individual metabolic cages, allowed free access to water and fresh diet, and maintained on a 12-h lights on/off cycle throughout the study.

At sacrifice, serum was collected and tissues (liver, heart, and skeletal muscle) immediately harvested into liquid nitrogen and stored at −70°C until analyzed.

Analyses. Serum free, acyl, and total carnitine were determined by a radioenzymatic method as described by Parvin and Pande (10). Tissues (liver, heart, and skeletal muscle) were prepared for carnitine (free, acyl, and total) and CAT activities determined as previously described (11,12). Carnitine was measured according to Paryin and Pande (10). Tissue carnitine results were expressed relative to DNA. DNA was determined according to the method of Labarca and Paigen (13).

CAT assay. The CAT activities were measured in 10% tissue (liver, heart, and muscle) homogenates by monitoring the conversion of [1-^{14}C]acetyl-CoA (50 μM) in the presence of carnitine (5 mM) to [1-^{14}C]acetyl-carnitine as described previously (10,14).

CPT assay. The activity of CPT was assayed in 10% tissue homogenates at 30°C with 50 μM [1-^{14}C]octanoyl-CoA as substrate in the presence of 5 mM carnitine according to Murthy and Pande (15). [1-^{14}C]Octanoyl-CoA was synthesized and purified as previously described (16).

Statistical analysis. The Student's *t*-test for unpaired data was used. Differences were considered significant at $P < 0.01$ for a two-tailed test.

RESULTS

Effect of HMG–CoA reductase inhibition on serum carnitine levels. Sixteen weeks of drug treatment significantly increased the serum free (24.0 ± 2.6 vs. 29.4 ± 3.1 μM), acyl (12.7 ± 3.1 vs. 26.5 ± 5.6 μM), and total (35.1 ± 4.7 vs. 52.8 ± 8.8 μM) carnitine concentrations in rabbit serum (Table 1).

Effect of HMG–CoA reductase inhibition on tissue carnitine levels. Sixteen weeks of feeding a diet containing lovastatin significantly decreased the tissue levels of carnitine in liver, heart, and skeletal muscle (Table 2). Among the tissues studied, skeletal muscle showed the greatest decrease in carnitine content.

Effects of HMG–CoA reductase inhibition on CAT and CPT activities in liver, heart, and skeletal muscle. The effects of drug treatment on the activities of CAT and CPT in liver, heart, and skeletal muscle are presented in Table 3. Treatment for 16 wk significantly increased the activities CPT in liver and heart (0.35 ± 0.09 vs. 0.50 ± 0.02 and 0.84 ± 0.22 vs. 1.33 ± 0.13 nmol · min^{-1} · g^{-1} DNA, respectively). Treatment had no effect on the activities of this enzyme in skeletal muscle. The CAT activity was unaffected by the treatment.

DISCUSSION

The pathogenic mechanisms underlying the myopathy and rhabdomyolysis that is occasionally seen with the use of HMG–CoA reductase inhibitors are unknown. Sixteen weeks of drug treatment in the rabbit significantly increased the serum levels of total carnitine. This increase was most evident in the concentrations of serum carnitine esters (Table 1). Of particular interest was that treatment significantly decreased the content of total carnitine in liver, skeletal muscle, and heart (Table 2). This observation may have implications

TABLE 1
Effects of 3-Hydroxy-3-Methylglutaryl-CoA Reductase Inhibition on Serum Levels of Carnitine in the Rabbit (μM)[a]

	Control	Treated
Free carnitine	24.0 ± 2.6	29.4 ± 3.1[b]
Acylcarnitine	12.6 ± 3.1	26.5 ± 5.6[b]
Total carnitine	35.1 ± 4.7	52.8 ± 8.8[b]
% Acylated	34 ± 7.6	48 ± 4.9[b]

[a]Values represent the mean ± SD ($n = 6$).
[b]$P < 0.01$; control vs. treated.

TABLE 2
Effects of 3-Hydroxy-3-Methylglutaryl–CoA Reductase Inhibition on Tissue Levels of Carnitine in the Rabbit (μmol/g DNA)[a]

	Liver		Heart		Skeletal muscle	
	Control	Treated	Control	Treated	Control	Treated
Free carnitine	73.5 ± 15.8	51.4 ± 7.2	236.4 ± 53.2	207.7 ± 57.9	1135.8 ± 303.8	653.0[b] ± 95.0
Total carnitine	87.1 ± 14.4	56.6[b] ± 7.7	468.3 ± 98.0	325.3[b] ± 79.0	1470.6 ± 326.7	894.2[b] ± 162.2

[a]Values represent the mean ± SD ($n = 6$).

TABLE 3
Effects of 3-Hydroxy-3-Methylglutaryl–CoA Reductase Inhibition on Tissue Levels of Carnitine Acetyltransferase (CAT) and Carnitine Palmitoyltransferase (CPT) Activities in the Rabbit (μmol · min^{-1} · g^{-1} DNA)[a]

	Liver		Heart		Skeletal muscle	
	Control	Treated	Control	Treated	Control	Treated
CAT	1.06 ± 0.15	0.97 ± 0.05	3.01 ± 0.77	3.64 ± 0.28	0.87 ± 0.28	0.89 ± 0.28
CPT	0.35 ± 0.09	0.50[b] ± 0.04	0.84 ± 0.22	1.33[b] ± 0.13	0.20 ± 0.09	0.29 ± 0.14

[a]Values represent the mean ± SD ($n = 6$).
[b]$P < 0.01$; control vs. treated.

with respect to the pathogenesis underlying the reported side effect of myalgia/myositis that occasionally occurs with this class of agents. Conceivably, over time, carnitine may be released from these tissues in the form of acylcarnitine as a mechanism by which the cells rid themselves of potentially toxic acyl-groups that may be accumulating as a by-product of mitochondrial metabolism. The continued release of acylcarnitine from the tissues may lead to a secondary carnitine deficiency over time. Carnitine is an important intracellular buffer for maintaining the acyl-CoA/CoASH ratio within cells (7,17,18).

Skeletal muscle is particularly sensitive to a deficiency state involving carnitine. Deficiency impacts type 1 fibers most markedly. Lipid is a major energy source of this fiber type both at rest and during prolonged exercise (19). As a carnitine deficiency develops, muscle long-chain acyl-CoA esters will divert to esterification reactions rather than oxidation. Depending upon the severity of the deficiency, this may result in the deposition of lipid droplets in muscle, giving rise to a lipid storage myopathy. In a less severe state, one might see a myositis and impaired muscle function secondary to the accumulation of acyl-CoA moieties and a decreased availability of free CoA for other reactions. Lipid storage myopathies secondary to carnitine deficiency have been reported with inherited deficiencies of different chain length acyl-CoA dehyrogenases (20–22), respiratory chain defects (23), methymalonic aciduria (24), and others. In all of these cases, the excretion of acylcarnitine increases with a concomitant rise in the [acylcarnitine]/[carnitine] ratio in plasma. Coincident with this, tissue levels of total carnitine decrease (25). A similar pattern was evident in the current study (Tables 1 and 2).

Rat liver HMG–CoA reductase and the fatty acid oxidation enzyme, CPT, are closely interlinked on a metabolic basis (26). It has been reported that chronic feeding of rats with an HMG–CoA reductase inhibitor increases the activities of liver outer mitochondrial CPT. More recently, it was observed that the rate of hepatic palmitate oxidation and the production of acid-soluble by-products were significantly increased with lovastatin treatment of rat hepatocytes (27). It was suggested that the underlying mechanism for this increase in fatty acid oxidation was due to an induction of hepatic CPT activity by the drug. The main acid-soluble product formed during the oxidation of palmitic acid by rat liver and muscle mitochondria is acetyl-CoA, which is readily esterified to carnitine forming acetylcarnitine. This esterification reaction is catalyzed by CAT (28). Once formed, acetylcarnitine is transported from the tissues to the plasma, where it is either metabolized or excreted unaltered by the kidneys. Our results of a significant increase in CPT activities in rabbit liver and heart after sixteen weeks of lovastatin treatment (Table 3) confirmed these findings. We did not see an effect of this drug on CAT activity. This may be due to the high activities of this enzyme that are already present in these tissues, making further induction unlikely. It is of interest to note that tissue levels of carnitine were decreased in all of the tissues studied, and CPT activity was increased in all those tissues, except in the case of skeletal muscle. The activity of CPT in skeletal muscle is not readily inducible under normal circumstances other than through exercise training; whereas in the liver and heart, the activity of this enzyme tends to respond more acutely to metabolic challenges.

In summary, we have shown that 16 wk of treatment with an HMG–CoA reductase inhibitor significantly alters the carnitine status in the rabbit. Further studies are necessary to establish whether or not these changes are functionally significant.

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Dietary Sunflower Oil Reduces Plasma and Liver Triacylglycerols in Fasting Rats and Is Associated with Decreased Liver Microsomal Phosphatidate Phosphohydrolase Activity

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ABSTRACT: Plasma and liver lipids were studied in male weanling rats fed diets containing moderate levels of fat (6% by weight) as sunflower oil (SF diet, rich in linoleic acid), salmon oil (SM diet, rich in long-chain n-3 fatty acids), or a blend of peanut and rapeseed oil (PR diet, rich in oleic acid). After nine weeks of feeding, the fasting plasma cholesterol concentrations were 49 and 24% lower in groups SM and SF, respectively, as compared to group PR. Both dietary salmon oil and sunflower oil lowered the triacylglycerol concentration of plasma and liver but, unexpectedly, the response was higher with sunflower oil. Indeed, in group SM the values were 15 and 30% lower in plasma and liver, whereas in group SF, they were 24 and 53% lower, respectively. As compared to group PR, liver triacylglycerols and microsomes contained 2.5- and 2.3-fold less oleic acid, respectively, in group SF, and they were 9.2- and 3.2-fold enriched in n-3 fatty acids, respectively, in group SM. The liver triacylglycerol concentrations were correlated with changes in the microsomal Mg^{2+}-dependent phosphatidate phosphohydrolase activity ($r = 0.47$, $P < 0.01$). As oleic acid, unlike long-chain n-3 fatty acids, is considered to promote the triacylglycerol synthesis and secretion, our findings suggest that changes in the membrane fatty acid composition could affect the triacylglycerol content of liver and plasma. Moreover, the availability within the liver, of oleic acid, predominantly incorporated into triacylglycerols, might limit the triacylglycerol production in SF-fed rats.

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In a recent study (1) we observed that in rats fed moderate levels of fats, dietary sunflower oil (SF) efficiently reduced fasting serum triacylglycerol levels, whereas salmon oil (SM) lowered serum cholesterol but had no significant reducing effect on triacylglycerols. These findings contrasted with numerous data showing that, in rats as well as in humans, fish oils rich in long-chain n-3 polyunsaturated fatty acids (PUFA) were the most active dietary fats to lower serum triacylglycerol levels (2–8). It has been demonstrated that the response was largely mediated by the inhibition of membrane-bound enzymes involved in triacylglycerol synthesis. Actually, long-chain n-3 PUFA reduce hepatic glycerophosphate acyltransferase (9–11), diacylglycerol acyltransferase (12–14), and phosphatidate phosphohydrolase (PAP) (15–19) activities. In contrast, oleic acid enhances the hepatocyte triacylglycerol synthesis and secretion (20–22). This effect may be explained by the capacity of oleic acid to activate the hepatic PAP (23,24) and to be preferentially utilized for triacylglycerol synthesis (22). Therefore, n-3 PUFA and oleic acid play a major role in the hepatic production of triacylglycerols by controlling the activity of PAP, which is a key enzyme of the synthetic pathway. In our previous study (1) oleic acid accounted for less than 10% of serum fatty acids in SF-fed rats. The concomitant alterations in membrane fatty acid composition suggested that the reduction of oleic acid in SF-fed rats, as well as the enrichment in long-chain n-3 PUFA in SM-fed rats, could contribute to lowering serum triacylglycerol levels by changing the activity of membrane-associated enzymes. Therefore, we have undertaken the present study to compare the responses of plasma and liver lipids to either dietary SF or SM. We have examined whether the reduction also concerned liver fractions, especially triacylglycerols, and whether dietary fats altered their oleic acid content. We have also determined whether fatty acid compositional modifications of a microsomal-enriched fraction were associated with changes in the Mg^{2+}-dependent PAP (EC 3.1.3.4) activity.

MATERIALS AND METHODS

Animals and diets. Male Wistar rats (IFFA CREDO, L'Arbresle, France) bred in our laboratory were randomly distributed into three groups of ten rats just after weaning (21-day-old). Animals were maintained at 22 ± 1°C with a 12-h light/dark cycle. During the following nine weeks, rats were fed *ad libitum* purified diets prepared in the laboratory, containing 6% by weight (about 13% of total calories) of different types of lipids.

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Abbreviations: DMPC, dimyristoylphosphatidylcholine; HDL, high density lipoprotein; LDL, low density lipoprotein; PA, Lα-phosphatidate; PAP, phosphatidate phosphohydrolase; PR, a blend of peanut oil and rapeseed oil; PUFA, polyunsaturated fatty acids; SF, sunflower oil; SM, salmon oil; VLDL, very low density lipoprotein.

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The experimental groups received either: (i) a mixture of peanut oil and rapeseed oil containing high levels of oleic acid and providing the required levels of PUFA (PR diet: n-6/n-3 = 5.2); (ii) SF (SF diet: n-6/n-3 = 664); (iii) SM (SM diet: n-6/n-3 = 0.10). The fatty acid composition of each diet is shown in Table 1.

The other dietary components were (w/w): 22% casein, 43.2% cornstarch, 21.6% sucrose, 4% mineral mixture, 1% vitamin mixture, 2% cellulose, and 0.16% methionine.

The mineral mixture contained (g/kg): Ca $HPO_4 \cdot 2\ H_2O$, 380; $K_2\ HPO_4$, 240; $CaCO_3$, 180; Mg $SO_4 \cdot 7\ H_2O$, 90; NaCl, 69; MgO, 20; $FeSO_4 \cdot 7\ H_2O$, 8.6; Zn $SO_4 \cdot H_2O$, 5; Mn $SO_4 \cdot H_2O$, 5; Cu $SO_4 \cdot H_2O$, 1; NaF, 0.8; CrK $(SO_4)_2 \cdot 12\ H_2O$, 0.5; KI, 0.04; $CoCO_3$, 0.02; $Na_2\ Se\ O_3 \cdot 5\ H_2O$, 0.02.

The vitamin mixture contained (g/kg triturated in sucrose): retinyl acetate (500 000 IU /g), 1; cholecalciferol (250 000 IU/g), 2.5; all-*rac*-α-tocopherol (500 IU/g), 10; menadione, 0.1; thiamin HCl, 1; riboflavin, 1; nicotinic acid, 4.5; calcium pantothenate, 3; pyridoxine HCl, 1; inositol, 5; D-biotin, 0.02; folic acid, 0.2; cyanocobalamin, 0.00135; ascorbic acid, 10; *p*-aminobenzoic acid, 5; choline chloride, 75.

After an overnight fast, rats were weighed and killed by decapitation without anesthesia and exsanguinated. Blood samples collected on EDTA (1 mg/mL) were centrifuged at low speed. Plasma was stored at −80°C after adding sodium azide (0.02%). Livers were rapidly removed, rinsed with ice-cold 150 mmol/L NaCl, blotted, and weighed. Aliquots were freeze-clamped and stored at −80°C.

Analytical procedures. Aliquot samples of thawed liver (corresponding to about 100 mg of fresh tissue) were homogenized in 4 mL of ice-cold buffer (150 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.4) using a loose-fitting Dounce homogenizer (Bioblock Scientific, Illkirch, France) with six up-and-down passes of the pestle. The protein content was determined according to Bradford (25) using the Biorad Protein assay (Biorad, Richmond, CA) with bovine serum albumin (fraction V) as standard. Lipids were extracted according to Folch *et al.* (26) using 6 mL of chloroform/methanol (2:1, vol/vol) for 1.5 mL homogenate. After two washes, the lower chloroform phase was dried under nitrogen and resolubilized in 0.25 mL of 2-propanol. Triacylglycerols and cholesterol (total and free) were quantified by enzymatic colorimetric tests (644200 and 1442341; Boehringer Mannheim, Meylan, France). The phospholipid content was estimated by the phosphorus content (P × 25) determined by the method of Bartlett (27).

Triacylglycerol and cholesterol concentrations were measured directly in plasma. High density lipoprotein (HDL)-cholesterol was estimated after precipitation of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) by the dextran sulfate Mg^{2+} procedure of Warnick *et al.* (28) adapted for rat plasma (29).

Preparation of liver membranes and metabolic assays. Liver tissue was rinsed with ice-cold NaCl 0.15 M, minced with scissors, and homogenized in 5 vol of buffer (250 mmol/L sucrose, 10 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA, 0.5 mmol/L dithiothreitol). After filtration through nylon gauze, the preparation was centrifuged at 20,000 × *g* for 20 min at 4°C. The supernatant (below the floating fat layer) was collected and centrifuged at 105,000 × *g* for 60 min at 4°C. The supernatant was collected as the cytosolic fraction (termed "cytosol") and the pellet as the microsomal enriched fraction (termed "microsomes").

The Mg^{2+}-dependent PAP activity was assayed by the method of Walton and Possmayer (30) with slight modifications. Liposomes were obtained by sonication (Labsonic 1510 Braun fitted with a microprobe; Labsonic Braun, Melsungen, Germany) in NaCl 150 mmol/L of equimolar (2 mmol/L) of Lα-phosphatidate (PA) and dimyristoylphosphatidylcholine (DMPC) (Sigma, St. Louis, MO).

The reaction mixture contained 0.5 mmol/L PA–DMPC liposomes, 1.5 mmol/$MgCl_2$ (16), and 50–100 μg microsomal protein in a final volume of 0.2 mL. The mixture was incubated at 37°C for 20 min. The reaction was terminated according to Mavis *et al.* (31) by adding 0.8 mL of a solution containing 0.13% SDS, 1.25% ascorbic acid, 0.32% ammonium molybdate, 4 H_2O, and 0.75 N H_2SO_4. The phosphomolybdate color was developed at 45°C for 20 min, and absorbance was measured at 820 nm. Cytosol was treated as described by Berglund *et al.* (32) using 0.23 g ammonium sulfate per mL solution for 10 min at 4°C. The precipitate was isolated after centrifugation at 10,000 × *g* for 10 min. The surface was gently washed with 50 mmol/L Tris-HCl pH 7.5, 230 mmol/L sucrose. It was then dissolved in the same buffer, and the PAP activity was determined.

Preliminary studies showed that the assays were linear with respect to both time of incubation and amount of protein.

TABLE 1
Fatty Acid Composition of Experimental Diets (wt%)

Fatty acid	PR diet[a]	SF diet[a]	SM diet[a]
14:0			7.0
16:0	8.2	7.2	15.3
16:1n-7	0.2		10.9
18:0	2.9	5.2	2.8
18:1n-9	58.6	20.3	15.0
18:1n-7	2.2	0.9	4.0
18:2n-6	22.4	66.4	2.6
18:3n-3	4.3	0.1	1.1
18:4n-3			2.8
20:1n-9	1.2		5.6
20:1n-11			3.4
20:4n-6			0.9
20:5n-3			13.4
22:5n-3			2.7
22:6n-3			12.6
Saturated	11.1	12.4	18.1
Monounsaturated	62.2	21.2	38.9
n-6	22.4	66.4	3.4
n-3	4.3	0.1	32.6
18:1n-9[b]	3516	1218	900
18:2n-6[b]	1344	3984	156
20:5n-3[b]			804
22:6n-3[b]			756

[a]Percentage of total. PR, in peanut and rapeseed oil blend; SF, sunflower oil; SM, salmon oil. No cholesterol in PR and SF diets; less than 0.05% in SM diet.
[b]mg/100 g Diet.

Fatty acid composition of liver lipids. Liver lipids were extracted according to Folch *et al.* (26) in the presence of butylated hydroxytoluene (0.02% wt/vol). Phospholipids were separated from neutral lipids on a silica cartridge (SEP-PACK; Waters Associates, Milford, MA) by successive elutions with chloroform, acetone, and methanol (33). Lipids were saponified in 15% KOH at 37°C for 5 h. The fatty acids were esterified by methanol containing 3% HCl and 4% 2,2 dimethoxypropane at 70°C for 35 min and analyzed by gas–liquid chromatography. The Carlo Erba model 4180 (Milan, Italy) chromatograph included an automatic injector on the column, a flame-ionization detector, and a CP wax 52 CB (carbowax 20 M) bonded fused silica capillary column (50 m × 0.3 mm i.d.). The assays were carried out with programmed oven temperature rises of 3°C/min from 54 to 200°C. Hydrogen was the carrier gas at a pressure of 0.8 bar. Peak areas were measured with an integrator connected to a microcomputer which expressed the data automatically. Peaks were identified by comparing their equivalent chain lengths with those of authentic fatty acid methyl esters.

Analysis of data. Data are expressed as the mean ± SD. Differences between dietary groups were determined by analysis of variance. Comparisons were made using Fisher's least significance difference test at the $P < 0.05$ level of significance.

RESULTS

There were no significant differences between groups for the mean final body weight (group PR 245.5 ± 14.3g ; group SF 231.9 ± 24.6 g; group SM 246.6 ± 22.4 g). Likewise, the liver protein contents were not altered by dietary treatments. The mean values ranged from 128 to 132 mg/g fresh liver. Table 2 shows the effect of dietary fatty acids on fasting plasma and liver lipids. SM, as well as SF feeding, significantly reduced plasma triacylglycerol concentrations compared with PR feeding. However, the effect was higher in group SF (–24%) than in group SM (–15%). Plasma cholesterol levels were significantly lowered, but more markedly in group SM (–49%) than in group SF (–24%). Nevertheless, dietary 18:2n-6 and long-chain n-3 PUFA had similar effects on plasma cholesterol vehicled either in VLDL + LDL or in HDL, as the HDL-cholesterol to total cholesterol ratio was not altered (0.80–0.83 range).

The triacylglycerol concentrations were also lowered in liver. Actually, both dietary sources of PUFA were very effective in significantly reducing the values, but the response was higher with SF (–53%) than with SM (–30%). The other lipid classes were moderately or not affected by dietary PUFA. Only SM significantly reduced phospholipids (–15%). Both SF and SM diets lowered liver cholesterol in the same proportion (10–12%), but they did not significantly change the unesterified to total cholesterol ratio (0.86–0.88 range). The fatty acid distribution in liver lipid classes is reported in Table 3. The major changes concerned monounsaturated and PUFA. The oleic acid content of triacylglycerols was lowered by 59% in group SF and by 47% in group SM compared to group PR. As expected, the triacylglycerols from SF-fed rats were enriched in n-6 PUFA, 18:2 (2.4-fold), and 20:4 (2.8-fold), whereas n-3 PUFA accounted for only 0.6% of total fatty acids. The response to dietary SM was also very pronounced. The 2.6-fold reduction of n-6 PUFA affected both 18:2 and 20:4 at similar extents, whereas the levels of long-chain n-3 PUFA were 9.2-fold higher than in group PR, with more than half of them as 22:6. Liver phospholipids were less affected than triacylglycerols. It is noticeable that SF feeding did not alter the proportion of 20:4n-6, whereas it increased that of 22:5n-6 by 14-fold. The increase in the n-6 pentaene to n-3 hexaene ratio likely reflected an n-3 deficiency (34). Conversely, SM feeding enhanced the long-chain n-3 PUFA content by 2.7-fold. This was compensated by the fall of n-6 PUFA (about threefold). A very minor fraction of liver acyl chains was used to esterify cholesterol. The major part of acyl chains consisted of palmitic acid (25–31%) and oleic acid (22–41%). Cholesteryl esters from SF-fed rats which did not contain long-chain n-3 PUFA were enriched by more than twofold in 18:2n-6. In SM-fed rats, the lowered content of

TABLE 2
Plasma and Liver Lipids in Fasting Rats After Nine Weeks of Dietary Treatments[a]

	PR diet	SF diet	SM diet
Plasma[b]			
Triacylglycerol	0.86 ± 0.12	0.65 ± 0.13[a]	0.77 ± 0.17[a]
Total cholesterol	1.66 ± 0.29[a]	1.26 ± 0.25[b]	0.84 ± 0.14[c]
HDL-cholesterol	1.35 ± 0.24[a]	1.04 ± 0.21[b]	0.68 ± 0.10[c]
HDL-C/total cholesterol	0.82 ± 0.05	0.83 ± 0.06	0.80 ± 0.07
Liver[c]			
Triacylglycerol	4.95 ± 1.60	2.34 ± 1.00[a]	3.45 ± 1.33[a]
Phospholipid	31.06 ± 3.12[a]	30.5 ± 1.08[a]	26.42 ± 1.77
Total cholesterol	5.31 ± 0.51	4.67 ± 0.35[a]	4.79 ± 0.54[a]
Free cholesterol	4.53 ± 0.39[a]	4.13 ± 0.28[b]	4.19 ± 0.50[a,b]
Free/total cholesterol	0.86 ± 0.05	0.88 ± 0.04	0.88 ± 0.06

[a]Data are expressed as means ± SD ($n = 10$). Means with no common superscript are significantly different at $P < 0.05$. Abbreviations as in Table 1; HDL, high density lipoprotein; C, cholesterol.
[b]μmol/mL.
[c]μmol/g.

TABLE 3
Fatty Acid Composition of Esterified Lipids in Rat Liver After Nine Weeks of Dietary Treatment[a]

Fatty acid	Triacylglycerol			Phospholipid			Cholesteryl ester		
	PR diet	SF diet	SM diet	PR diet	SF diet	SM diet	PR diet	SF diet	SM diet
16:0	23.7 ± 1.6[a]	23.1 ± 1.0[a]	26.6 ± 0.9	17.1 ± 0.5[a]	17.6 ± 1.4[a]	20.8 ± 0.6	25.7 ± 2.8	30.6 ± 3.8[a]	31.0 ± 2.1[a]
16:1n-9	0.8 ± 0.2	0.4 ± 0.1[a]	0.4 ± 0.03[a]	0.1 ± 0.02[a]	0.1 ± 0.1[a,b]	0.1 ± 0.1[b]	1.9 ± 0.9	2.8 ± 2.6	2.1 ± 0.8
16:1n-7	2.7 ± 0.5[a]	1.6 ± 0.3[b]	6.1 ± 0.6[c]	0.7 ± 0.1[a]	0.5 ± 0.1[b]	1.5 ± 0.1[c]	4.3 ± 0.8[a]	2.7 ± 0.5[b]	11.3 ± 2.0[c]
18:0	2.5 ± 0.5[a]	3.0 ± 0.4[b]	2.9 ± 0.2[a,b]	20.3 ± 2.8	24.9 ± 1.3[a]	24.1 ± 0.8[a]	5.4 ± 1.1[a]	7.2 ± 1.8	5.4 ± 0.9[a]
18:1n-9	43.1 ± 2.7[a]	17.5 ± 1.7[b]	23.0 ± 1.2[c]	4.3 ± 0.4	2.8 ± 0.8[a]	3.0 ± 0.3[a]	41.1 ± 4.0[a]	22.2 ± 3.3[b]	27.9 ± 2.2[c]
18:1n-7	2.9 ± 0.2	2.0 ± 0.2[a]	2.1 ± 0.2[a]	2.4 ± 0.3	1.9 ± 0.2[a]	2.0 ± 0.1[a]	2.1 ± 0.2[a]	1.8 ± 0.2[b]	3.1 ± 0.3[c]
18:2n-6	16.3 ± 1.6[a]	39.0 ± 2.0[b]	5.8 ± 1.1[c]	6.8 ± 1.1[a]	9.0 ± 1.0[b]	2.2 ± 0.3[c]	10.0 ± 1.1[a]	22.4 ± 4.2[b]	6.1 ± 3.2[c]
18:3n-3	1.0 ± 0.2[a]	0.1 ± 0.1[b]	0.7 ± 0.1[c]	nd	nd	nd	0.9 ± 0.1[a]	0.2 ± 0.1[b]	0.6 ± 0.2[c]
20:1n-9	0.4 ± 0.1[a]	0.2 ± 0.1[b]	0.7 ± 0.1[c]	0.2 ± 0.05[a]	0.1 ± 0.05[b]	0.3 ± 0.05[c]	0.1 ± 0.1[a]	nd	0.5 ± 0.1[b]
20:3n-9	0.3 ± 0.1	0.2 ± 0.1	nd	0.3 ± 0.03	nd	0.2 ± 0.02	nd	0.1 ± 0.1	nd
20:3n-6	0.1 ± 0.1[a]	0.3 ± 0.1[b]	0.2 ± 0.02[c]	0.5 ± 0.1[a]	0.2 ± 0.04	0.5 ± 0.05[a]	0.1 ± 0.1[a,b]	0.1 ± 0.1[a]	0.1 ± 0.02[b]
20:4n-6	3.6 ± 0.5[a]	10.3 ± 1.4[b]	1.5 ± 0.2[c]	33.9 ± 1.7[a]	34.2 ± 1.1[a]	11.4 ± 0.8[b]	7.7 ± 1.4[a]	9.4 ± 2.3[b]	3.3 ± 0.6[c]
20:5n-3	0.4 ± 0.3[a]	nd	9.2 ± 1.4[b]	0.2 ± 0.04[a]	nd	8.7 ± 1.0[b]	0.2 ± 0.02[a]	nd	6.5 ± 1.3[b]
22:1n-9	nd	nd	nd	nd	nd	nd	0.01 ± 0.01	0.2 ± 0.1	nd
22:4n-6	0.2 ± 0.1[a]	1.0 ± 0.2[b]	nd	0.3 ± 0.04[a]	1.2 ± 0.1[b]	nd	nd	0.4 ± 0.1	nd
22:5n-6	0.1 ± 0.1[a]	1.0 ± 0.2	0.2 ± 0.02[a]	0.3 ± 0.1[a]	4.8 ± 0.5[b]	0.2 ± 0.1[a]	nd	nd	nd
22:5n-3	0.3 ± 0.2[a]	nd	4.1 ± 0.4[b]	0.7 ± 0.1[a]	0.2 ± 0.1[b]	3.4 ± 0.3[c]	nd	nd	0.6 ± 0.1
22:6n-3	1.6 ± 0.7[a]	0.5 ± 0.2[b]	16.5 ± 1.4[c]	11.8 ± 1.1[a]	2.5 ± 0.3[b]	21.7 ± 1.4[c]	0.2 ± 0.2[a]	nd	1.4 ± 0.2[b]
Saturated	26.2 ± 1.8[a]	26.1 ± 0.9[a]	29.5 ± 0.9	37.4 ± 2.8	42.5 ± 0.7[a]	44.9 ± 0.5[a]	31.1 ± 3.8	37.9 ± 5.5[a]	36.4 ± 2.8[a]
Monounsaturated	49.9 ± 2.6[a]	21.6 ± 2.0[b]	32.3 ± 1.1[c]	7.7 ± 0.7[a]	5.3 ± 0.9[b]	6.8 ± 0.4[c]	49.7 ± 4.0[a]	29.6 ± 3.7[b]	44.9 ± 3.3[c]
n-6	20.3 ± 2.1[a]	51.5 ± 2.8[b]	7.7 ± 1.3[c]	41.8 ± 1.9[a]	49.5 ± 0.7[b]	14.3 ± 1.2[c]	17.8 ± 0.8[a]	32.3 ± 4.9[b]	9.5 ± 3.1[c]
n-3	3.3 ± 1.2[a]	0.6 ± 0.5[b]	30.5 ± 2.0[c]	12.7 ± 1.1[a]	2.7 ± 0.3[b]	33.8 ± 1.1[c]	1.3 ± 0.3[a]	0.2 ± 0.1[b]	9.1 ± 1.5[c]

[a]Data are expressed as mol per 100 mol of the total fatty acids and are means ± SD (n = 10). For any lipid class, means with no common superscript are significantly different at $P < 0.05$. Abbreviations as in Table 1; nd, not detected or lower than 0.1%.

n-6 PUFA was replaced mainly by 20:5n-3 rather than 22:6. In contrast, 22:6 was the predominent n-3 PUFA in the other ester classes.

Figure 1 represents the liver content of fatty acyl chains incorporated in the three classes of esterified lipids. None of the fatty acids present in hepatic cholesteryl esters exceeded 0.3 μmol/g. Accordingly, almost all liver fatty acids were distributed between phospholipids and triacylglycerols. Irrespective of dietary fats, liver phospholipids from the three groups had similar amounts of saturated and monosaturated fatty acids, which could originate from both endogenous and exogenous sources. Palmitic acid was distributed in the three classes, whereas stearic acid was almost entirely found in phospholipids. Irrespective of dietary treatments, phospholipids contained more than 12 μmol 18:0/g liver vs. 0.2–0.4 and 0.04–0.07 in triacylglycerols and cholesteryl esters, respectively. Oleic acid was the major fatty acid of triacylglycerols. The highest content, obtained with the PR diet, reached 6.4 μmol/g liver. The values were 2.7- and 5.2-fold lower in groups SM and SF, respectively. Because of the lowering effect of dietary SF on liver triacylglycerol levels, the amounts of 18:2n-6 incorporated in liver triacylglycerols were only scarcely higher in group SF than in group PR (2.7 vs. 2.4 μmol/g liver, respectively). The phospholipid content of liver was not influenced by SF feeding. Accordingly, both PR and SF groups incorporated similar amounts of 20:4n-6 in this lipid class. The value, 21 μmol/g liver, represented about 96% of 20:4n-6 present in the liver-esterified lipids. More than 70% of 20:4n-6 was replaced by 20:5 and 22:6n-3 in phospholipids from group SM. The amounts reached 4.6 and 11.4 μmol/g liver, respectively.

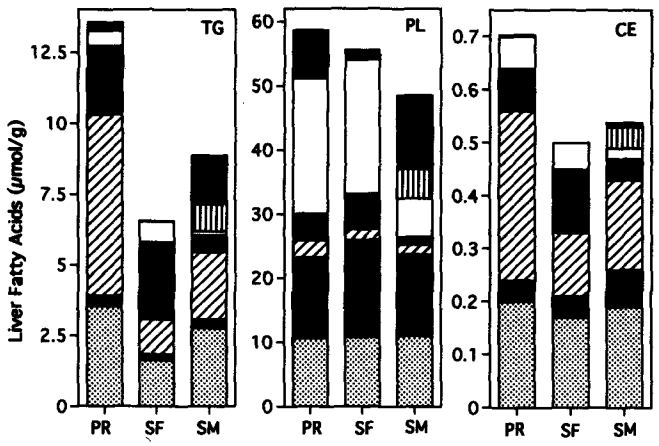


FIG. 1. Amounts of fatty acids in liver-esterified lipids (μmol/g fresh weight). Values were calculated using data from Tables 2 and 3. Triacylglycerols (TG), phospholipids (PL), and cholesteryl esters (CE) contained 3, 2, and 1 mol fatty acid/mol ester, respectively. Esterified cholesterol was total cholesterol minus free cholesterol. Dotted bar, 16:0; black bar, 18:0; white cross-hatched bar, 18:1n-9; grey bar, 18:2n-6; white bar, 20:4n-6; vertically striped bar, 20:5n-3; and gray cross-hatched bar, 22:6n-3; PR, a blend of peanut oil and rapeseed oil; SF, sunflower oil; SM, salmon oil.

The fatty acid composition of the microsomal fractions resembled that of total phospholipids, with more 18:1n-9 and 18:2n-6 and less 22:6n-3 in the three dietary groups (Table 4). The mean values from all the three groups showed a significant positive correlation between the liver triacylglycerol concentrations and the percentage of oleic acid in either liver triacylglyc-

TABLE 4
Fatty Acid Composition of a Microsomal-Enriched Fraction of Rat Liver After Nine Weeks of Dietary Treatment[a]

Fatty acid	PR diet	SF diet	SM diet
16:0	19.7 ± 0.6[a]	18.6 ± 0.4[b]	25.6 ± 1.4[a]
16:1n-7	0.9 ± 0.1[a]	0.5 ± 0.1[b]	2.3 ± 0.2[c]
18:0	22.3 ± 0.5[a]	24.0 ± 0.5[b]	20.9 ± 1.9[c]
18:1n-9	10.2 ± 0.6[a]	4.4 ± 0.3[b]	8.0 ± 0.5[c]
18:1n-7	2.4 ± 0.1[a]	1.9 ± 0.2	2.5 ± 0.1[a]
18:2n-6	8.6 ± 0.3[a]	13.0 ± 0.5[b]	3.9 ± 0.4[c]
18:3n-3	0.2 ± 0.02	nd	nd
20:3n-9	0.2 ± 0.02[a]	0.2 ± 0.03[a]	0.1 ± 0.01
20:3n-6	0.4 ± 0.1[a]	0.3 ± 0.02[b]	0.6 ± 0.04[c]
20:4n-6	26.8 ± 0.7[a]	31.0 ± 0.9[b]	10.6 ± 0.6[c]
20:5n-3	0.3 ± 0.04[a]	nd	8.1 ± 0.9[b]
22:4n-6	0.2 ± 0.02[a]	1.0 ± 0.1[b]	nd
22:5n-6	0.2 ± 0.03[a]	3.5 ± 0.2[b]	0.2 ± 0.03[a]
22:5n-3	0.6 ± 0.1[a]	0.2 ± 0.02[b]	2.5 ± 0.3[c]
22:6n-3	6.8 ± 0.2[a]	1.4 ± 0.2[b]	14.5 ± 1.6[c]
Saturated	42.0 ± 0.7[a]	42.6 ± 0.5[a]	46.5 ± 3.1[b]
Monounsaturated	13.6 ± 0.7[a]	6.8 ± 0.4[b]	12.8 ± 0.7[c]
n = 6	36.3 ± 0.7[a]	48.8 ± 0.7[b]	15.3 ± 0.9[c]
n-3	7.8 ± 0.3[a]	1.5 ± 0.2[b]	25.2 ± 2.4[c]
UI	4.5	4.4	4.5

[a]Data are as described in Table 3; mol/100 mol. Abbreviations as in Table 1; UI (unsaturation index) = sum $(a \times b)/S$ where a is the relative percentage of each unsaturated fatty acid, b is the number of double bonds for that particular fatty acid, and S is the percentage of total saturated fatty acids.

TABLE 5
Effects of Dietary Lipids on Mg^{2+}-Dependent Phosphatidate Phosphohydrolase (PAP) Activity in Rat Liver[a]

Pap activity	PR diet	SF diet	SM diet
Cytosol	7.77 ± 1.31[a]	6.47 ± 1.29[b]	4.92 ± 0.95[c]
Microsomes	5.38 ± 0.64[a]	4.21 ± 0.94[b]	4.3 ± 0.74[b]
% Of total in microsomes	41 ± 5[a]	39 ± 5[a]	47 ± 6[b]

[a]The PAP activity in either cytosol or microsomes was estimated by the release of inorganic phosphate from phosphatidate-dimyristoylphosphatidylcholine liposomes in the presence of 1.5 mmol/L Mg^{2+}. Data are as described in Table 2; nmol/min/mg protein. Abbreviations as in Table 1.

erols ($r = 0.97$; $P < 0.05$) or in liver microsomes ($r = 0.97$; $P < 0.05$).

The activity of liver Mg^{2+}-dependent PAP (Table 5) was greater in cytosol than in microsomes. Feeding PUFA decreased the activity in both fractions, but the values differed between groups SF and SM only in cytosol, as the activities were lowered by 17 and 37%, respectively. Pooled data from the three groups showed a significant and positive correlation between liver triacylglycerol concentrations and microsomal PAP activity ($r = 0.47$; $P < 0.01$) (Fig. 2). There was no correlation between plasma triacylglycerol concentrations and phosphatidate hydrolysis.

DISCUSSION

The present results confirm our recent data showing the higher capacity of SF as compared to SM to lower plasma triacylglycerol levels in fasting rats (1). The same protocol was used for both experiments, but in the present study neither plasma nor liver samples were pooled. They were treated individually in order to obtain a more accurate response to dietary treatments. This may explain that, unlike previous results, the difference between plasma triacylglycerols levels from groups PR and SM was significant.

The response of plasma cholesterol to dietary PUFA partially depends on that of plasma triacylglycerols. Indeed, a fraction of cholesterol is delivered to plasma in VLDL, the triacylglycerol-rich particles further converted into LDL. However, other mechanisms might explain the reduction of HDL-cholesterol as this fraction represented at least 80% of total plasma cholesterol. Our results agree with those of Balasubramaniam *et al.* (3) showing that, in rats, fish oil was more efficient than safflower oil in decreasing the total plasma cholesterol levels. These authors suggested that n-3 and, to a lesser extent, n-6 PUFA increased the secretion of cholesterol into bile. They hypothesized that the effects could be related to an accelerated uptake of lipoproteins by the liver, possibly due to alterations in the plasma membrane of liver cells.

The phosphatidate hydrolysis was different in the three dietary groups. However, neither total nor soluble PAP activity

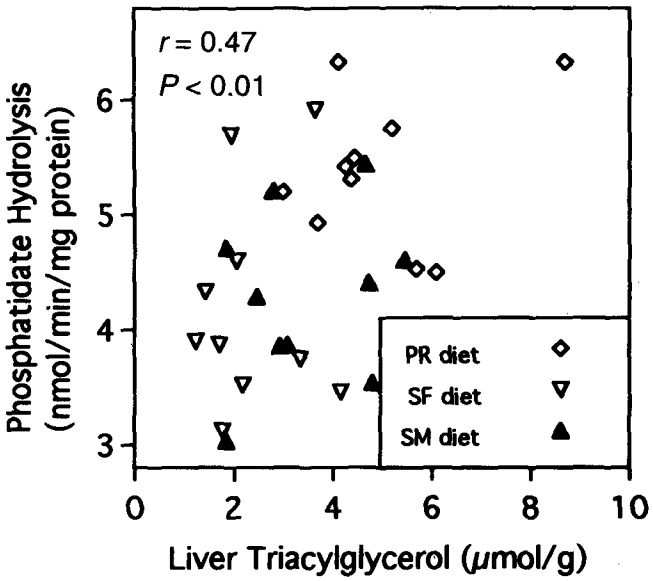


FIG. 2. Correlation between microsomal Mg^{2+}-dependent phosphatidate phosphohydrolase activity and triacylglycerol concentrations of liver in rats fed different dietary lipids for nine weeks (n = 10/dietary group). Values from Table 5 are plotted vs. those from Table 2. See Figure 1 for abbreviations.

was correlated with plasma or liver triacylglycerol levels. The 24% lower cytosolic PAP activity in group SM compared to group SF was of the same magnitude as that obtained in three-week-old rats fed diets containing either fish oil or SF (18). It is recognized that long-chain fatty acids control the expression of PAP (23). In rat hepatocytes, oleate increases the PAP activity by promoting the translocation of the enzyme from the cytosol to the endoplasmic reticulum (24). This was not observed in this work as the fraction of total activity in microsomes was not higher in group PR than in the other groups. Likewise, no change was observed either in a study using rat liver perfused with either oleic acid or long-chain n-3 PUFA (16), or in feeding experiments comparing responses to vegetable and fish oils (17,35). The values found for microsomal activity were probably overestimated because inorganic phosphate was produced *via* phosphatidate deacylation and dephosphorylation (36). Moreover, plasma membranes present in the microsomal fraction contain a PAP not involved in triacylglycerol synthesis (37).

Anyway, the positive correlation of liver triacylglycerol concentrations with phosphatidate hydrolysis in the microsomal fraction of liver supports the hypothesis that dietary-induced changes in the microsomal fatty acid distribution could alter the expression of the bound PAP. Our data showing the reducing effect of dietary long-chain n-3 PUFA on the Mg^{2+}-dependent microsomal PAP activity are consistent with results obtained in rats (15) and Syrian hamsters (19). *In vitro* studies on liver (12) and intestinal (14) cells showed that microsomal membranes enriched in eicosapentaenoic acid synthesized less triacylglycerols than membranes enriched in oleic acid. Hence the difference between responses to PR, SF, and SM diets might be due partly to changes in the eicosapentaenoic and oleic acid contents of liver microsomes. In this study, liver microsomes contained less oleic acid in group SF than in group SM. In contrast, Balasubramaniam *et al.* (3) found the same proportion of oleic acid (9%) in liver microsomes of weanling rats fed either fish oil or safflower oil, but only fish oil lowered plasma triacylglycerol levels. This might be linked to the higher intake of oleic acid than in our study (2 g/100 g diet). The 57% lower microsomal proportion of oleic acid in group SF compared with group PR was partly due to the inhibitory effect of linoleic acid on the desaturation of stearic acid as shown by other authors. In weanling Wistar rats, replacing dietary olive oil by corn oil lowered the oleic acid content of liver microsomes by 66%. This corresponded to opposite changes in Δ6 and Δ9 desaturase activities (38). Like in our experiment, the value of unsaturation index was practically unchanged. This contributed to maintaining the homeostasis of membranes (38,39).

Another cause of the reduced triacylglycerol levels in liver might be the lowering of the pool of oleic acid available for esterification. This assumption is supported by the positive correlation found between the percentage of oleic acid in liver triacylglycerols and the triacylglycerol content of liver. Moreover, we previously observed that serum lipids incorporated less oleic acid in group SF than in group SM (9 vs. 16), despite a higher intake (1).

Although a fraction of oleic acid has an endogenous origin, the amounts present in liver may be restricted because dietary PUFA have the capacity to reduce the hepatic synthesis of fatty acids (40,41) and to stimulate the mitochondrial oxidation of long-chain fatty acids. The latter effect has been demonstrated for the n-3 series (10,11,19) but not for the n-6 series. Moreover, fish oil can induce a reduction in plasma free fatty acids which contributes to lowering of hepatic triacylglycerols (42). Few studies showing the more potent reducing effect of diets rich in fish oil compared to diets rich in vegetable oil on plasma triacylglycerol levels report the responses of liver triacylglycerol levels. Yeo and Holub (43) found lower amounts in fish oil- than in SF-fed rats. The discrepancy with our study could be related to the higher intake of fats (10%), which provided higher amounts of oleic acid for triacylglycerol synthesis. This assumption is supported by the presence of equivalent high proportions (21%) of oleic acid in liver triacylglycerols from both dietary groups. Similar differences between liver triacylglycerol levels were observed in rats fed 15% (9) and 20% (35) lipid diets. The response to diets rich in fish oil was also due to the stimulation of peroxisomal fatty acid oxidation.

In contrast with our study, Rustan *et al.* (35) showed much higher amounts of monoenes in liver triacylglycerols with a sunflower oil diet (2.52 instead of 1.52 μmol/g liver) than with a fish oil diet (1.79 instead of 3.34 μmol/g liver). This could reflect a higher rate of triacylglycerol synthesis in SF-fed rats because diets provided high amounts of oleic acid, preferentially incorporated into triacylglycerols. The effect of SF cannot be assigned to n-6 PUFA because 18:2n-6 only possesses a weak hy-

potriglyceridemic activity, whereas 20:4n-6 may elevate circulating triacylglycerols (44). We observed that SM, but not SF, reduced liver phospholipids. Indeed, the replacement of decreased 20:4n-6 is limited (45) despite the high capacity of long-chain n-3 PUFA to be incorporated into phospholipids (14,19,22).

In summary, the present study shows that under our experimental conditions dietary SF had a tendency to be more efficient than dietary SM to lower both plasma and liver triacylglycerols. In particular, intake of dietary lipids was lower than in many other studies. It is obvious that several mechanisms are involved in the responses to dietary PUFA. Among them, our data underlines the role of membrane fatty acids in the rate of hepatic phosphatidate hydrolysis. The lack of stimulation by oleic acid, as well as the inhibition by long-chain n-3 PUFA of the esterification pathway, might contribute to the hypotriglyceridemic effect of SF and SM, respectively. The concomitant reduction of oleic acid in liver triacylglycerols and microsomes suggests that the availability of oleic acid might limit the triacylglycerol production in SF-fed rats.

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Effect of a Synthetic Androgen on Biliary Lipid Secretion in the Female Hamster

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ABSTRACT: This study was designed to elucidate the effect of the synthetic androgen, methyltestosterone, on bile flow and biliary lipid secretion in female hamsters. Animals were divided into four groups and fed the following diets: group 1, lithogenic diet for three weeks; group 2, lithogenic diet + 0.05% methyltestosterone for three weeks; group 3, lithogenic diet for six weeks; group 4, lithogenic diet + 0.05% methyltestosterone for six weeks. At the end of each experimental period, the hamsters were operated on to establish external biliary fistulas. During the depletion of the endogenous bile acid pool (for two hours), the basal bile flow of group 4 was significantly smaller than that of group 3. Basal bile acid output was significantly lower in the methyltestosterone-fed groups 2 and 4 than in control groups 1 and 3. In contrast, groups 2 and 4 secreted more cholesterol than groups 1 and 3. Group 4 had a higher ratio of cholesterol output to phospholipid output than group 3. Increasing doses of taurocholate were infused after the bile acid depletion period, and it was found that methyltestosterone did not change the bile acid independent bile flow. The increments in cholesterol or phospholipid output induced per increment of bile acid output (linkage coefficients) were analyzed by linear regression. The methyltestosterone-fed groups (groups 2 and 4) had a higher linkage coefficient of cholesterol output to bile acid output than the control groups (groups 1 and 3). The linkage coefficients of phospholipid output to bile acid output of groups 2 and 4 were also higher compared to groups 1 and 3. The linkage coefficient of cholesterol output to phospholipid output of group 2 was higher than that of group 1. These results suggest that methyltestosterone stimulated the cosecretion mechanism of cholesterol and phospholipid in bile associated with an increasing ratio of cholesterol to phospholipid. In conclusion, the synthetic androgen, methyltestosterone, caused a decrease in basal bile flow and bile acid secretion, and an increase in basal cholesterol secretion and the biliary cholesterol-to-phospholipid ratio. These findings explain, in part, how methyltestosterone intensifies the formation of cholesterol gallstones in female hamsters. *Lipids 31*, 879–886 (1996).

In humans, the incidence of cholesterol gallstones is about twice as high in females as in males (1). Oral contraceptives (2,3) and estrogen therapy in postmenopausal women (4), as well as prostate cancer treatments (5), raise the risk of developing gallstones in these subjects. Such observations strongly suggest that, in humans, female sex hormones promote gallstone formation, but the mechanism of this effect is not understood. The effects of male sex hormones on gallstone disease have not been studied in detail. In previous studies, we found that, unlike humans, male hamsters from Sasco fed a lithogenic diet (LD, containing 4% butterfat and 0.3% cholesterol) had a high incidence of cholesterol gallstones (up to 90%), while females of the same "strain" formed no stones even when palmitic acid was substituted for butterfat to enhance the lithogenic stimulus (6–8). However, when these female hamsters were fed the LD containing 0.05% methyltestosterone (MeT), a synthetic androgen, they developed cholesterol gallstones as readily as the males (9). MeT administration to female hamsters increased the biliary cholesterol saturation index, raised the proportion of biliary cholesterol carried in vesicles, and changed the biliary bile acid composition to that of the males (9). The male hormone appeared to stimulate the formation of lithogenic bile and cholesterol gallstones; it is unclear whether MeT exerted this effect by stimulating biliary cholesterol secretion, inhibiting bile acid secretion, or modifying phospholipid secretion. In order to elucidate the mechanism of action of MeT, we fed an LD containing this androgen to female hamsters and studied bile flow and biliary lipid secretion.

MATERIALS AND METHODS

Animals and diets. Fifty-eight female golden Syrian hamsters (42–56 g) were purchased from Sasco, Inc. (Omaha, NE). The hamsters were divided into four groups and fed the LD with or without MeT (Dyets, Inc., Bethlehem, PA) and water *ad libitum* for three or six weeks prior to surgery: group 1 ($n = 11$), LD for three weeks; group 2 ($n = 15$), LD + 0.05% MeT for three weeks; group 3 ($n = 16$), LD for six weeks; and group 4 ($n = 16$), LD + 0.05% MeT for six weeks. The LD contained 43.6% corn starch, 20.0% casein, 14.6% dyetrose (soluble starch), 10.0% fiber (cellulose), 5.0% salt mix (modified U.S.P. XIV salt mix No. 200951), 4.0% butterfat, 2.0% corn oil, 0.5% vitamin mix (No. 300000), and 0.3% cholesterol.

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Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; EE, ethinylestradiol; LD, lithogenic diet; LKC, linkage coefficient; MeT, methyltestosterone; TC, taurocholic acid.

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Surgical procedures. The hamsters were anesthetized by intraperitoneal injection of 20 mg/100 g body weight of ketamine (Ketaset; Fort Dodge Laboratories, Inc., Fort Dodge, IA) at the end of each feeding period. Additional intramuscular injections of ketamine (10 mg) were used to maintain anesthesia. A polyethylene catheter (PE-10, 0.28 mm i.d., Clay Adams, Parsippany, NJ) was inserted into the femoral vein, and 0.9% saline was infused at a rate of 1.13 mL/h using a Harvard syringe pump (Harvard Apparatus, Millis, MA). After laparotomy, the cystic duct was ligated with a hemostatic clip and the common bile duct was cannulated using polyethylene tubing (PE-10). Saline infusion was maintained for two hours to deplete the endogenous bile acid pool. Sodium taurocholate (TC) (Sigma Chemical Co., St. Louis, MO) was then infused at increasing rates of 1 and 6 μmol/h/100 g body weight together with trace amounts of 24-^{14}C-labeled TC (Amersham Life Science, Arlington Heights, IL); each dose of TC was infused for two hours. In the six-week groups, TC infusion was increased up to 18 μmol/h/100 g body weight to evaluate a potential cholestatic effect of MeT on bile acid secretion. Bile samples were collected every 20 min in preweighed vials and stored at −20°C until analyzed. At the end of the experiment, the gallbladder was removed and examined for gallstones.

Analytical procedures. The presence of cholesterol gallstones and crystals in the gallbladder was confirmed by polarizing light microscopy (Olympus MCHAP microscope; Olympus Corp., Lake Success, NY). For measurement of the recovery of [^{14}C]TC, 10 μL of hepatic bile was counted in a Beckman LS 3801 liquid scintillation system (Beckman Instruments, Fullerton, CA) with automatic quench correction, using Aquasol-2 (NEN Research Products, Boston, MA) as the scintillation fluid. Bile volumes were determined gravimetrically, and the samples were divided into aliquots for analysis of the biliary lipids. Total bile acid concentration was measured enzymatically using a commercial kit (# 450-A; Sigma Chemical Co.); the bile acid composition was further analyzed by gas–liquid chromatography after preparation of the methyl ester acetate derivatives, as previously reported (10). The phospholipid concentration was determined enzymatically (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The cholesterol concentration was measured by gas–liquid chromatography after formation of the trimethylsilylether derivative (11).

Calculations and statistics. The differences in the incidence of cholesterol gallstones and biliary cholesterol crystals were calculated by the Fisher exact test (12). Bile flow was expressed as microliters per minute per kilogram body weight; biliary lipid output was expressed as micromoles per minute per kilogram body weight. The basal bile flow and the biliary lipid output during depletion of the endogenous bile acid pool were calculated using the data of the 60–120 min period before TC infusion. We excluded the data of the initial 0–40 min, as bile flow and biliary lipid secretion were unstable during the first 40 min. The data are presented as the mean ± SD. The means were compared by analysis of variance to determine the F statistic. Student's t-test was then used for values for which the F statistic was significant ($P < 0.05$) (12). The relationships between bile flow and bile acid output, cholesterol output and bile acid output, phospholipid output and bile acid output, and cholesterol output and phospholipid output were analyzed by linear regression for the period of bile acid depletion and TC infusion (1 to 6 μmol/h/100 g body weight). The regression lines were plotted; the y-intercepts and slopes were calculated. The linkage coefficient (LKC) is defined as the slope of the regression line, that is, the increment in cholesterol or phospholipid output induced per increment of bile acid output, and the incremental ratio of cholesterol output to phospholipid output (13–15). Comparisons were made between the slopes and intercepts of the groups receiving the LD vs. LD + MeT (group 1 vs. group 2, group 3 vs. group 4) by using SAS/STAT software (version 6.10; SAS Institute Inc., Cary, NC) (16,17). The groups fed LD + MeT for three and six weeks were also compared (group 2 vs. group 4). A difference was considered significant at $P < 0.05$.

RESULTS

The hamsters gained weight and remained healthy during administration of the experimental diets. Table 1 summarizes the incidence of cholesterol gallstones and crystals at the end of three and six weeks. No stones were found in groups 1 and 3, which received the LD without MeT. The addition of MeT to the diet induced cholesterol gallstones: at the end of three weeks, two hamsters (13%) had gallstones and four hamsters (27%) had biliary cholesterol crystals (group 2), while at the end of six weeks, 13 hamsters (81%) had gallstones and crystals (group 4).

TABLE 1
Incidence of Cholesterol Gallstones and Cholesterol Crystals at Sacrifice[a]

Group	Diet	Feeding period (wk)	Cholesterol gallstones [no. of animals (%)]	Cholesterol crystals [no. of animals (%)]
1	LD	3	0/11 (0)	0/11 (0)
2	LD + MeT	3	2/15 (13)	4/15 (27)
3	LD	6	0/16 (0)	1/16 (6)
4	LD + MeT	6	13/16[b,c] (81)	13/16[b,c] (81)

[a]Animals were fed a lithogenic diet (LD) with or without methyltestosterone (MeT) for three or six weeks. See the Materials and Methods section for details.
[b]Differs from group 2; $P < 0.01$.
[c]Differs from group 3; $P < 0.01$.

At the end of the feeding periods, surgery was performed to establish a biliary fistula and insert the femoral vein cannula. TC was infused i.v. at increasing doses after depletion of the endogenous bile acid pool. When the maximum dose (6 μmol/h/100 g body weight for the 3-wk groups and 18 μmol/h/100 g body weight for the 6-wk groups) was infused, the recovery of TC was 102.6 ± 4.5, 102.7 ± 6.8, 98.7 ± 6.6%, and 98.0 ± 19.6% for groups 1–4, respectively. There were no significant differences between the control group and the MeT group for both feeding periods. Figure 1 illustrates the time course of bile flow at the end of three weeks (A) and at the end of six weeks (B). Groups 2 and 4, which were fed LD + MeT, secreted less bile than the corresponding groups on LD (groups 1 and 3) during the 120-min bile acid depletion period. During TC infusion, there were no significant differences in bile flow between the control groups and the hormone-treated groups (group 1 vs. 2, group 3 vs. 4). The time course of biliary bile acid secretion in each group paralleled the rate of bile flow (data not shown). Figure 2 illustrates the time course of biliary cholesterol secretion. At the end of three weeks (Fig. 2A), the animals fed MeT (group 2) secreted more cholesterol during depletion of the bile acid pool; this difference ceased after TC infusion. At the end of six weeks (Fig. 2B), the MeT group (group 4) had a consistently higher cholesterol output than the control group (group 3) for the entire period.

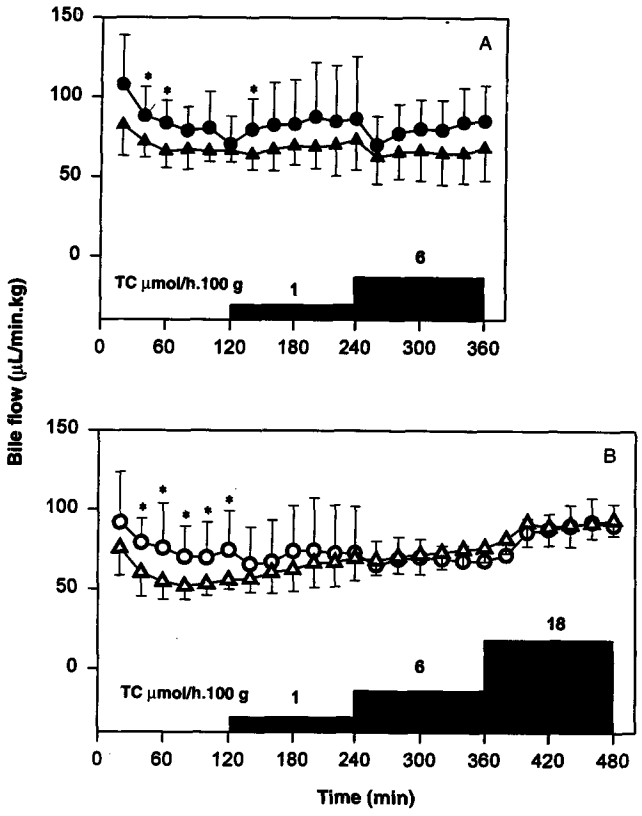


FIG. 1. Time course of bile flow during bile acid depletion and taurocholic acid (TC) infusion periods. A: group 1, lithogenic diet (LD) for three weeks (●) and group 2, LD + 0.05% methyltestosterone (MeT) for three weeks (▲). B: Group 3, LD for six weeks (○) and group 4, LD + 0.05% MeT for six weeks (△). Values represent the means ± SD. *Significant differences were determined by Student's *t*-test, $P < 0.05$.

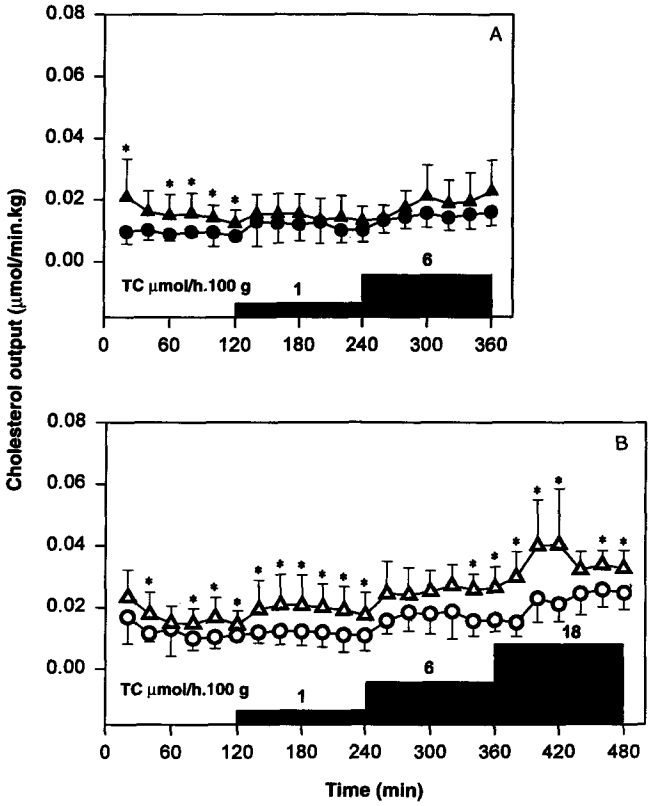


FIG. 2. Time course of cholesterol output during bile acid depletion and TC infusion periods. A: Group 1, LD for three weeks (●) and group 2, LD + 0.05% MeT for three weeks (▲). B: Group 3, LD for six weeks (○) and group 4, LD + 0.05% MeT for six weeks (△). Values represent the means ± SD. *Significant differences were determined by Student's *t*-test, $P < 0.05$. See Figure 1 for abbreviations.

Bile acid analysis of the hepatic bile before TC infusion showed that the administration of MeT altered the bile acid composition. The percentage of cholic acid (CA) in the MeT-fed groups was significantly lower than in the LD controls. At the end of three weeks, the percentage of CA was 50.5 ± 1.8% in group 1 and 40.6 ± 4.1% in group 2 ($P < 0.02$). At the end of six weeks, the percentage of CA was 43.8 ± 4.6% in group 3 and 29.1 ± 2.0% in group 4 ($P < 0.01$). As a result, the ratio of CA/chenodeoxycholic acid (CDCA) was lower in the bile of the MeT-treated animals (3.2 ± 1.0 vs. 1.4 ± 0.4, group 1 vs. group 2, $P < 0.05$; and 2.0 ± 0.6 vs. 0.8 ± 0.1, group 3 vs. group 4, $P < 0.01$). During TC infusion (at a rate of 6 μmol/h/100 g body weight), CA accounted for about 90% of biliary bile acids in all groups. The CA/CDCA ratio was 48.4, 26.2, 42.1, and 28.7 for groups 1–4, respectively.

Table 2 summarizes basal bile flow, biliary lipid output, and the ratio of cholesterol output to phospholipid output during depletion of the endogenous bile acid pool. The six weeks MeT-treated group (group 4) had the smallest bile flow. In the three weeks groups, basal bile flow of group 2 was less than group 1, but this difference was not statistically significant.

TABLE 2
Basal Biliary Lipid Secretion During Depletion of the Endogenous Bile Acid Pool[a]

Group number	Bile flow (μL/min/kg)	BA output (μmol/min/kg)	CH output (μmol/min/kg)	PL output (μmol/min/kg)	Ratio of CH output/PL output
1	78.4 ± 17.5	0.281 ± 0.083	0.009 ± 0.003	0.084 ± 0.068	0.291 ± 0.314
2	66.5 ± 9.1	0.194 ± 0.075[b]	0.014 ± 0.005[b]	0.120 ± 0.060[b]	0.133 ± 0.046
3	72.8 ± 23.0	0.287 ± 0.128	0.011 ± 0.005	0.096 ± 0.073	0.407 ± 0.466
4	54.1 ± 8.2[c,d]	0.171 ± 0.057[d]	0.015 ± 0.005[d]	0.055 ± 0.060[c,d]	0.956 ± 0.871[c,d]

[a]Data were used for the 60–120-min period before taurocholate infusion. See the Materials and Methods section for details of statistical analysis; BA, bile acid; CH, cholesterol; PL, phospholipid.
[b]Differs from group 1; $P < 0.01$.
[c]Differs from group 2; $P < 0.01$.
[d]Differs from group 3; $P < 0.02$.

Basal bile acid output was significantly lower in the hormone-treated animals as compared to the control animals (group 2 vs. group 1, group 4 vs. group 3). In contrast, cholesterol secretion was significantly higher in the MeT-fed animals as compared to the control animals. Phospholipid output was variable in each animal; the MeT-treated animals showed an increase after three weeks, but a decrease at the end of six weeks. The ratio of cholesterol output to phospholipid output increased significantly at the end of six weeks in the MeT-treated hamsters (group 4 vs. group 3).

Table 3 summarizes the coefficients of the linear regression equations describing the relationships between bile flow and biliary lipid outputs. The effect of MeT on the relationship between bile flow and bile acid output is also shown in Figure 3. The *y*-intercept is defined as the bile acid independent bile flow, and the slope represents the bile acid dependent bile flow (μL/μmol of bile acid) (18). MeT appeared to decrease the bile acid dependent bile flow (slope) after three and six weeks, but these values were not statistically significant (Fig. 3, Table 3). In the MeT-fed animals, the bile acid independent bile flow (intercept) decreased after a longer feeding period (group 2 vs. group 4). There were no differences between intercepts of control groups and MeT groups (group 1 vs. group 2, group 3 vs. group 4). The relationship between cholesterol output and bile acid output was altered by MeT (Fig. 4, Table 3). At the end of three weeks, LD + MeT (group 2) caused a greater increment of cholesterol output per 1 μmol of bile acid output, (LKC, slope), than LD alone (group 1). After six weeks, the stimulatory effect of MeT on cholesterol output remained; the LKC of group 4 was higher than that of group 3. MeT feeding exerted a similar relationship for phospholipid output and bile acid output. The LKC of phospholipid output to bile acid output of groups 2 and 4 was higher than that of groups 1 and 3, respectively (Table 3). The LKC of cholesterol output to phospholipid output in group 2 was higher than that in group 1 and decreased after six weeks (group 4) (Table 3).

DISCUSSION

In previous studies, we observed a relationship between gender and stone formation in the hamster, but the mechanism by which this occurred was not clear (8,9). We postulated that a hormone produced changes in the secretion of one or more of the three biliary lipids. In the present study, we used the female hamsters fed LD with or without MeT in an attempt to elucidate the mechanism(s) involved. Bile acid infusion to replace the endogenous bile acids allowed us to probe for relationships between bile flow, cholesterol secretion, phospholipid secretion, and bile acid secretion. In humans (19,20), dogs (20), and rats (21,22), a hyperbolic relationship was reported between cholesterol output and bile acid output, and between phospholipid output and bile acid output. However, Gurantz and Hofmann (14) and Berr *et al.* (15) showed that in the hamster, under the conditions employed, these relationships appeared to be almost linear. In our study, the relationships between the secretion of the three biliary lipids were also compatible with linear functions; consequently, we ana-

TABLE 3
Coefficients of Linear Regression Equations for Relation Between Bile Flow, Bile Acid, Cholesterol, and Phospholipid Outputs[a]

Group number	Bile flow vs. BA output			CH output vs. BA output			PL output vs. BA output			CH output vs. PL output		
	Slope	Intercept	*r*	Slope	Intercept	*r*	Slope	Intercept	*r*	Slope	Intercept	*r*
1	64.5	61.3	0.39	0.020	0.006	0.56	0.160	0.046	0.39	0.035	0.009	0.39
2	39.0	61.2	0.52	0.040[b]	0.007	0.73	0.267[b]	0.046	0.75	0.102[b]	0.006[b]	0.67
3	51.4	53.4	0.39	0.024	0.006	0.56	0.192	0.048	0.43	0.023	0.011	0.24
4	35.2	54.1[c]	0.35	0.039[d]	0.012	0.48	0.364[c,d]	0.010	0.58	0.030[c]	0.018[c]	0.23

[a]Linear regression lines were plotted using the data of bile acid depletion and taurocholate infusion (1 to 6 μmkol/h 100 g body weight) period; coefficients were calculated from linear regression lines. See the Materials and Methods section for details of statistical analysis. See Table 2 for abbreviations.
[b]Differs from group 1; $P < 0.025$.
[c]Differs from group 2; $P < 0.02$.
[d]Differs from group 3; $P < 0.01$.

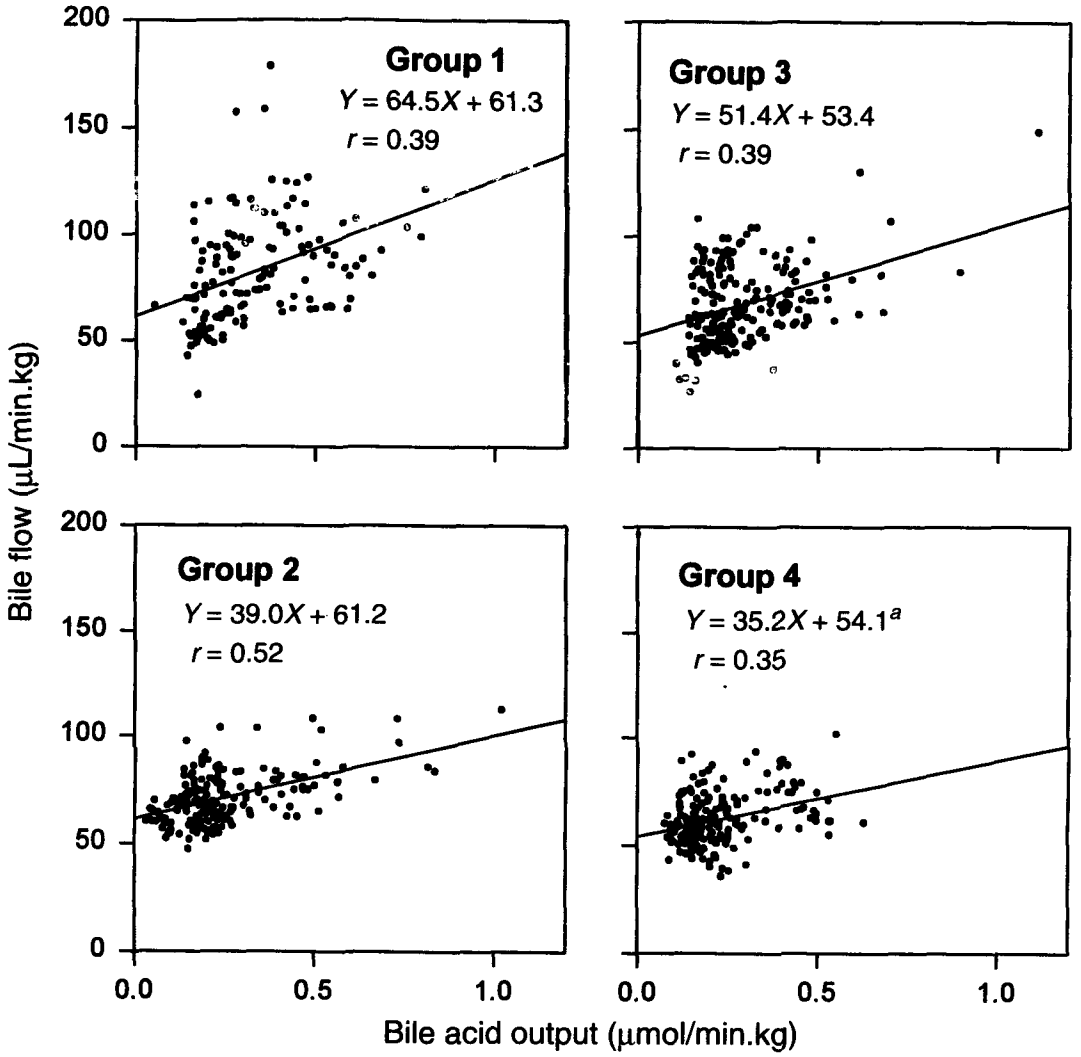


FIG. 3. Relationships between bile flow and bile acid output during bile acid depletion and TC infusion periods (1–6 µmol/h.100 g body weight). Group 1, LD for three weeks; group 2, LD + 0.05% MeT for three weeks; group 3, LD for six weeks; group 4, LD + 0.05% MeT for six weeks. Coefficients of the linear regression equations were compared among pertinent groups (group 1 vs. group 2, group 3 vs. group 4, and group 2 vs. group 4). [a]Differs from group 2; $P < 0.02$. See Figure 1 for abbreviations.

lyzed the relationships by linear regression (Figs. 3, 4; Table 3). Apparently, there are species differences in the mechanism of biliary lipid secretion.

We examined, for the first time, the effects of a synthetic androgen on bile flow and biliary lipid secretion. MeT reduced basal bile flow during depletion of the bile acid pool (Fig. 1; Table 2). However, this androgen did not inhibit the excretion of radiolabeled TC at the maximum rate of TC infusion (6 and 18 µmol/h/100 g body weight after 3 and 6 wk, respectively). There were no differences in the bile acid independent bile flow between control groups and MeT groups. These results showed that MeT was less cholestatic than certain estrogens; the latter have been reported to decrease bile acid independent bile flow (23–26). MeT seemed to decrease basal bile flow by reducing basal bile acid secretion. TC infusion restored bile acid secretion and bile flow in group 4 to the level of those of the control group (group 3) (Fig. 1). MeT might reduce the secretion of bile acids by decreasing bile acid synthesis, as it did not inhibit the recovery of labeled TC (greater than 98%). Davis *et al.* (27) showed that ethinylestradiol (EE) reduced the activity of cholesterol 7α-hydroxylase, the rate-limiting enzyme of bile acid synthesis, by increasing the viscosity of the microsomal membrane. Similar studies using androgens have not been reported.

MeT altered biliary lipid secretion. This hormone decreased the basal output of bile acids and increased cholesterol output (groups 2 and 4). The LKC of cholesterol output to bile acid output and phospholipid output to bile acid output increased in the hamsters fed MeT. These changes resulted in the production of lithogenic bile and formation of cholesterol gallstones. There are several possible explanations for the effects of MeT on biliary lipid secretion. First, changes in the

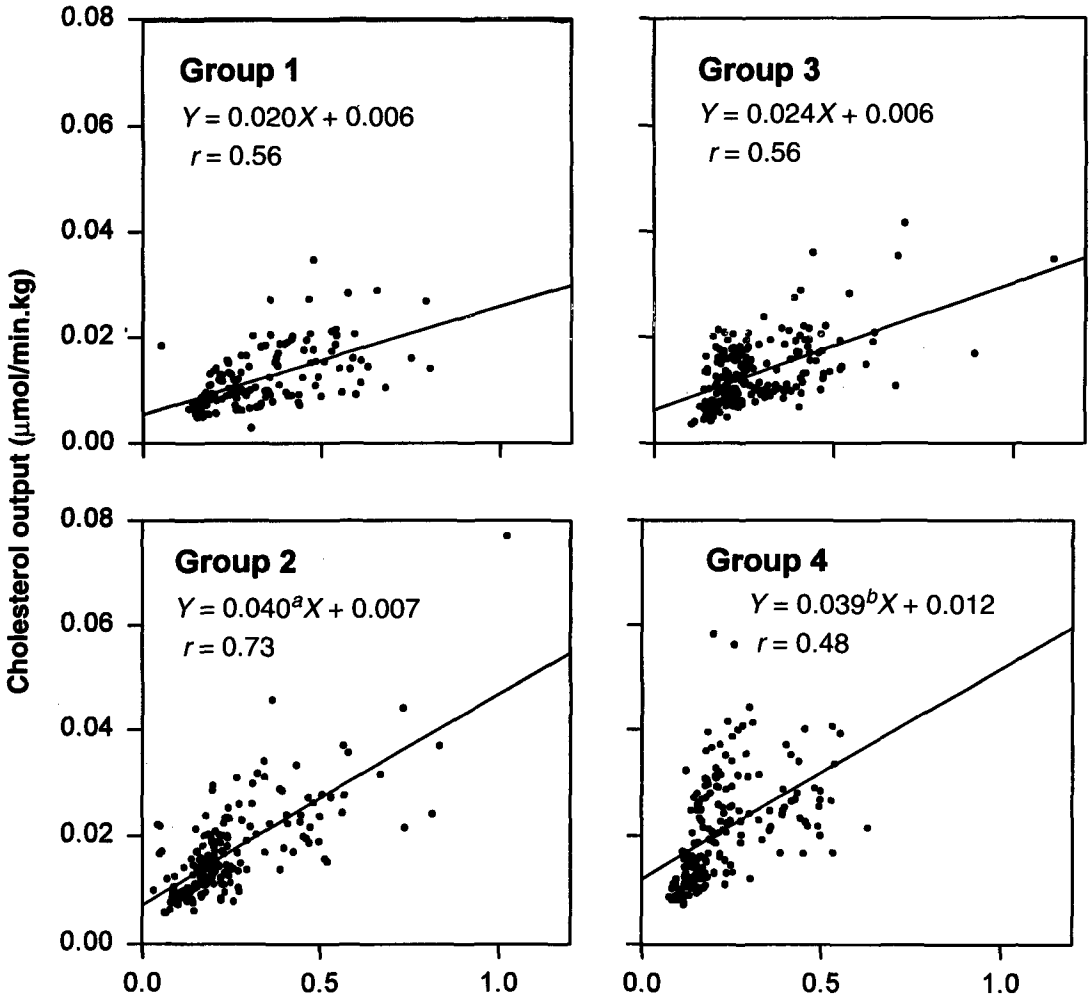


FIG. 4. Relationships between cholesterol output and bile acid output during bile acid depletion and TC infusion periods (1–6 μmol/h.100 g body weight). Group 1, LD for three weeks; group 2, LD + 0.05% MeT for three weeks; group 3, LD for six weeks; group 4, LD + 0.05% MeT for six weeks. Coefficients of linear regression were compared among pertinent groups (group 1 vs. group 2, group 3 vs. group 4, and group 2 vs. group 4). [a]Differs from group 1; $P < 0.025$, [b]differs from group 3; $P < 0.01$. See Figure 1 for abbreviations.

biliary bile acid composition by the hormone could alter the secretion of cholesterol and phospholipid in bile. MeT decreased the CA/CDCA ratio in bile. It was previously observed that there was a sex difference in bile acid composition in the hamster (8,9,28). It is possible that MeT inhibited 7α-hydroxy-4-cholesten-3-one 12α-hydroxylase, thus reducing the ratio of CA/CDCA. In hamsters, CDCA was found to be more effective in stimulating the secretion of biliary cholesterol than CA (14). The change in the CA/CDCA ratio could be partly responsible for the increase in cholesterol secretion in the female hamsters fed MeT. However, this interpretation may only partially explain the stimulatory effect of MeT on cholesterol secretion. Hamsters fed MeT secreted more cholesterol than the control groups, even during a high dose infusion of TC (when the proportion of CA in bile was 90% in both groups) (Fig. 2). Second, hormones have been shown to produce a change of cholesterol distribution in the hepatocyte which might lead to an increase in biliary cholesterol secretion. The female sex hormone, EE, increased membrane cholesterol, resulting in reduced membrane viscosity (24,25,29,30). Another female hormone, progesterone, inhibited acyl coenzyme A/cholesterol acyltransferase and increased free cholesterol in the biliary cholesterol precursor pool, resulting in an increased secretion of cholesterol (21,23,31). Presumably, MeT may have increased the amount of cholesterol in the canalicular membrane or altered the free cholesterol compartment of the biliary cholesterol precursor pool to induce high cholesterol secretion in bile. Third, MeT may directly affect a presently unknown mechanism of biliary lipid secretion. Berr *et al.* (15) reported that EE increased cholesterol and phospholipid secretion without increasing bile acid output or changing the bile acid composition, possibly by this unknown mechanism of cholesterol secretion.

It is postulated that cholesterol and phospholipid are secreted together in vesicular form into the canaliculi (32,33). In the present study, the LKC of cholesterol to bile acid and phospholipid to bile acid were higher in the LD + MeT groups than in the LD groups. The LKC of cholesterol to phospholipid was also higher on LD + MeT at the end of three weeks. MeT increased the ratio of cholesterol output to phospholipid output during basal secretion at six weeks. These data suggested that MeT stimulated a cosecretion mechanism with an increasing ratio of cholesterol to phospholipid. A similar effect was reported for EE, which increased the secretion of cholesterol and phospholipid at a constant cholesterol/phospholipid ratio (15).

In conclusion, the synthetic androgen, MeT, decreased basal bile flow and bile acid secretion, stimulated basal cholesterol secretion, and raised the ratio of cholesterol to phospholipid in the bile of female hamsters. These changes led to the formation of cholesterol gallstones. The mechanism of gallstone formation is complex; a variety of factors besides biliary lipids must be considered. It is possible that MeT affected gallbladder motility, mucin production, nucleating/antinucleating proteins, the structure of the gallbladder epithelium, and/or phospholipid transfer protein(s) (34–38). However, the detailed mechanism whereby MeT induces changes in biliary lipid metabolism is still unknown.

ACKNOWLEDGMENTS

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Salivary Lipid Profiles of the Leech (*Hirudo medicinalis*)

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ABSTRACT: Saliva was collected from sixteen leeches (*Hirudo medicinalis*). The saliva was analyzed for its total lipid content (3.26 ± 0.31 mg of total lipids per 100 mL saliva). The lipids were separated into polar and nonpolar by chromatographic techniques. The neutral fraction was approximately 2/3 of the total, and the polar fraction was approximately 1/3 of the total lipids. Thin-layer chromatography was used to obtain the individual profiles of the polar and nonpolar lipids. Of the identified lipids, phosphatidic acids and free fatty acids represented the largest percentage. These results suggest that the leech contains a unique lipid distribution, and that some of these components may be potent phospholipases and lipases that probably are present in its saliva for the purpose of preventing plugging or healing of the wound in the attacked organism.
Lipids 31, 887–888 (1996).

Although some nonlipid components of the saliva of leeches (*Hirudo medicinalis*) have been reported, no information is available concerning the lipid profile of this important medicinal species. It was felt that the lipid content and composition may yield information that may be helpful in explaining the anticoagulatory properties found in leeches. Many leech products are currently used and many more are being investigated (1,2).

MATERIALS AND METHODS

Sixteen leeches (*H. medicinalis*) were purchased from commercial sources, and the salivary fluid was obtained by established methods (3–10). All lipids or solutions containing lipids were kept under nitrogen at all times. When stored, lipids were kept under nitrogen at −20°C in the presence of approximately 5 μg of butylated hydroxytoluene per mg of lipid. Lipid standards and plates were purchased from Applied Sciences (State College, PA). Each batch of saliva was individually extracted five times, one hour each time (one extraction followed the others) with 4 mL of chloroform/methanol (2:1, vol/vol) at 0°C. One aliquot of this abstract was subjected to silicic acid column chromatography to separate phospholipid (by methanol elution) from neutral lipids (by chloroform elution). Individual lipid families were obtained from the separated neutral lipids and phospholipids by chromatography on thin-layer plates, and each was individually identified and assayed. Silica Gel-G glass plates with petroleum ether/ethyl ether/acetic acid (90:10:1, by vol) was used for the nonpolar lipids. Silica Gel-H with chloroform/methanol/acetic acid/water (200:120:25:15, by vol) was used for the polar lipids (3,4).

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RESULTS AND DISCUSSION

The results from the lipid profiles were obtained from 16 individual analyses of leeches (Table 1). The lipid composition is very different from that observed in other species, and may be unique for this invertebrate. The total lipid content observed was approximately 3 mg per 100 mL of saliva. This is about half of the total lipids found in the saliva of many vertebrates. Although most vertebrates show a high content of phosphatidylcholine (4–7), the content of lecithin in the leech was exceedingly small. The content of phosphatidylserine and phosphatidylinositol was small and very similar to that of most species. The large amounts of phosphatidic acids found

TABLE 1
Lipid Analysis of Leech Saliva (16 specimens)

Total lipids (mg/100 mL)	3.26 ± 0.31[a]
Percentage of total lipid	
Neutral lipids	66.67 ± 12.4
Polar lipids	33.33 ± 7.8
Neutral lipid families	
Mono- + diglycerides	4.97 ± 2.1
Cholesterol	9.25 ± 2.3
Free fatty acids	17.02 ± 3.7
Triglycerides	13.47 ± 3.5
Methyl esters	8.09 ± 4.7
Cholesterol esters	10.71 ± 5.9
Unidentified	8.16
% Total neutral lipids	66.67
Polar lipid families	
Phosphatidic acid	18.80 ± 5.7
Cardiolipin	trace <.01
Phosphatidylethanolamine	3.02 ± 1.7
Phosphatidylinositol	1.97 ± 1.5
Phosphatidylserine	2.44 ± 1.9
Phosphatidylcholine	1.51 ± 1.7
Lysophosphatidylcholine	1.39 ± 1.3
Sphingomyelin	1.51 ± 1.8
Unidentified	2.69
% Total polar lipids	33.33

[a]Values are mean ± SEM.

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may indicate that the saliva of the leech contains very active phospholipases. These results also suggest that the leech must have various powerful lipases that could help hydrolyze any clots formed after the leech has attached itself to the skin. This ability is essential so that the leech may be able to prevent coagulation that would stop the loss of blood flow from the attacked individual (11,12).

The percentage of other minor components (sphingomyelin, lysolecithin, cardiolipin, etc.) of the polar lipid group is within the range found in most other species. The total polar lipid amount as a percentage of the total lipid content is a little above that of most other species. Nonpolar lipids in the leech represent approximately 2/3 of the total lipid content, which is a smaller amount than that found in most species. The percentage of cholesterol and cholesterol esters found represent a very small amount of lipid compared to that found in human saliva. The amount of mono- and diglycerides represent almost 5% of the total lipids recovered. This is a value much higher than the percentage observed in the other species examined (5–9). This may occur due to high nonpolar lipase activity in the saliva of the leech, which would be understandable considering the high phospholipase activity that was also observed.

These results suggest that the saliva of the leech has a lipid profile that is compatible with and necessary for its functions. These functions permit the easy flow of blood and of other nutritional products from the attacked individual and may help prevent the closing of the wound or coagulation that would impede the flow of fluids to the leech (13–15).

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ERRATUM

In *Lipids*, Vol. 31, no. 5 (May 1996), "An Improved Method for Rapid Analysis of the Fatty Acids of Glycerolipids," by Ken'ichi Ichihara, Akira Shibahara, Kohei Yamamoto, and Takao Nakayama, pp. 535–539, the right side of Figure 5 was inadvertently cropped off. The complete figure appears below.

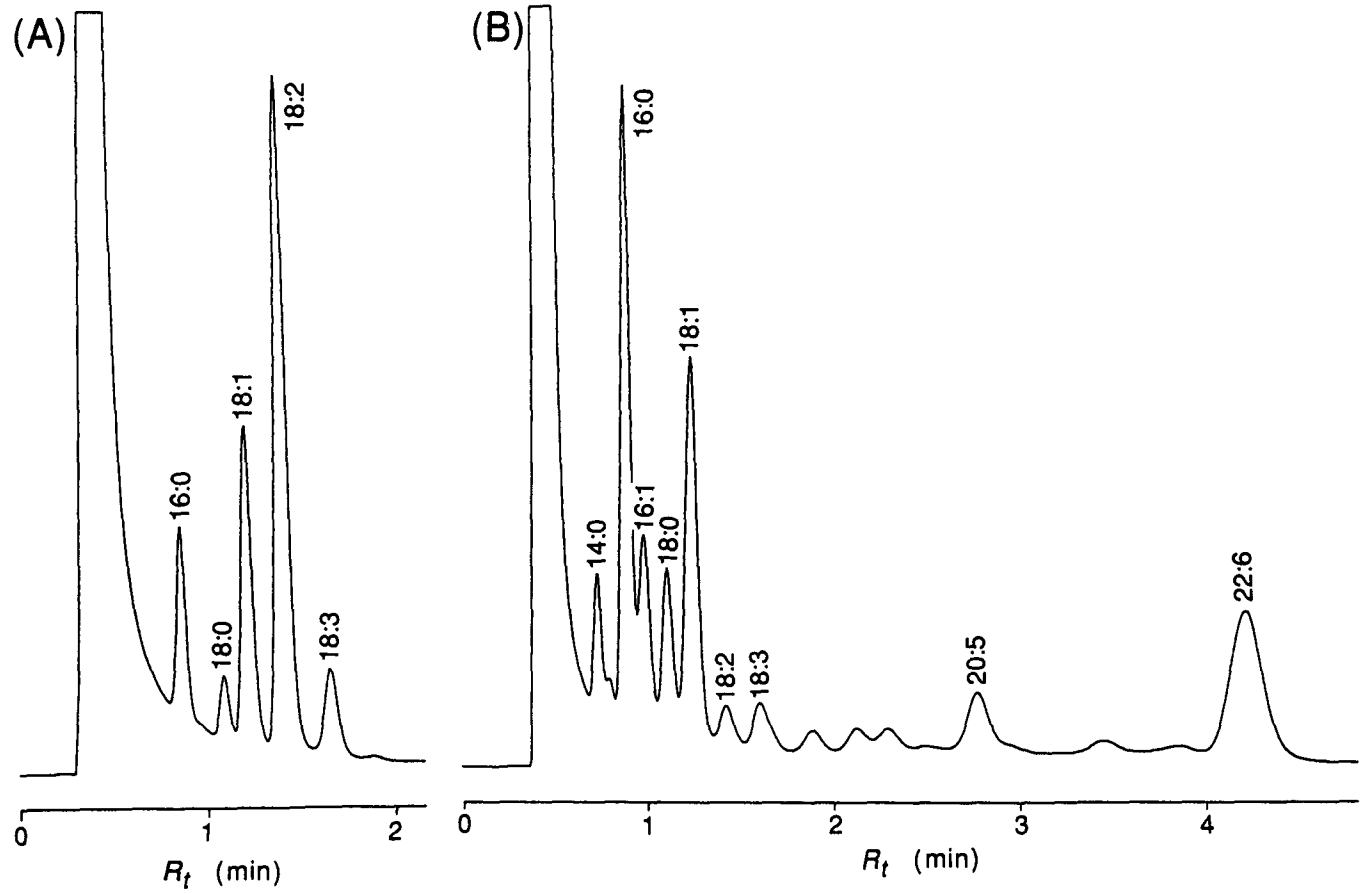


FIG. 5. High-speed gas chromatography (GC) of fatty acid methyl esters prepared from (A) soybean oil and (B) a 22:6-enriched fish oil. GC conditions: 3 mm × 2 m glass column; column packing, 10% SP-2340 on 100–120 mesh Chromosorb WAW; column temperature, 240°C; N_2, 40 mL/min.

ANNOUNCEMENTS

CALENDAR

September 1996

37th International Conference on the Biochemistry of Lipids, Antwerp University Center, University of Antwerp, Sept. 4–7, 1996. Symposia: enzymes and transfer proteins related to lipoprotein metabolism, aspects of protein lipidation, and peroxisomal lipid metabolism. Oral and poster communications covering the themes of the symposia and miscellaneous topics of the broad field of lipid biochemistry. Information: Professor Doctor A. Lagrou, RUCA-Lab Human Biochemistry, Department BFG, University of Antwerp, Groenenborgerlaan 171, B2020 Antwerp, Belgium (Phone: 3 2180329; Fax: 3 2180326).

7th International Congress on Phospholipids, Sept. 9–11, 1996, Brussels, Belgium. Program topic areas include Growth, Death and Cancer; Choline, Choline Phospholipids and the Brain; Importance of Phospholipids in Health and Disease. Information: AOCS Meetings Department, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

Training in Free Radical Methodologies, Sept. 23–27, 1996, CERLIB, University of Grenoble, Grenoble, France. Production, damages, repair: ESR, nitric oxide, DNA oxidation, DNA repair, apoptosis, chemical-trapping, lipoprotein oxidation, protein oxidation, cellular production and toxicity. Information: Arlette Alcaraz, Laboratoire de Biochimie C, CHU Grenoble BP 217, 38043 Grenoble Cédex 9, France (Phone: 33 76 76 57 54; Fax: 33 76 76 56 64; E-mail: CERLIB@ujf-grenoble.fr).

October 1996

Frontiers in Lipid and Lipoprotein Research: Basic Science, Analytical, Clinical, and Public Health Applications, Oct. 4–6, 1996, Stouffer Renaissance Hotel, Dallas, Texas. Sponsored by the Lipids and Lipoproteins Division of the American Association for Clinical Chemistry, the Centers for Disease Control and Prevention, and the American Heart Association. An integrated review of relevant recent advances in basic science, measurement technology, epidemiological and clinical research, and public health issues will be presented. This review will help define state-of-the-art techniques for understanding and preventing morbidity and mortality due to heart disease. Information: Paula M. Steiner, Medical Research Laboratories, 2 Tesseneer Dr., Highland Heights, KY 41076 (Phone: 606-781-8877, Extension 252; Fax: 606-781-9310; E-mail: lipf@cehehll.em.cdc.gov).

American College of Nutrition, 37th Annual Meeting, Oct. 11–13, 1996, San Francisco, California (Phone: 212-777-1037; Fax: 212-777-1103).

Certification Board for Nutrition Specialists, Oct. 11, 1996, San Francisco, California. Certifying examination for nutritionists and dieticians with advanced degrees. Information: CBNS, 1562 First Avenue, Box #258, New York, NY 10028 (Phone: 212-332-0774 (leave message); Fax: 212-777-1103).

Seminar: Interconversion of Polyunsaturated Fatty Acids, and Their Functions in Membranes, Oslo, Norway, Oct. 11–12, 1996. Information: Georg Lambertsen, Secretary, Lipidforum, Breistølen 1, N-5035, Bergen, Norway (Phone: 4755 + 32 89 56; Fax: 47 55 + 32 57 24).

"Confectionery Fats" Symposium, Belgrave Sq., London, Oct. 15–16, 1996, Oils and Fats Group—Society of the Chemical Industry, UK. Information: Secretariat, SCI, 14/15 Belgrave Sq., London.

November 1996

AOCS Conference on PUFA in Infant Nutrition: Consensus and Controversies, Nov. 7–9, 1996, Barcelona, Spain. Information: Susan E. Carlson (E-mail: scarlson@utmem1.utmem.edu); Robert A. Gibson (E-mail: rgibson@paed_nnu.fmc.flinders.edu.au); Berthold Koletzko (Fax: 49-89-5160-4733); or AOCS Meetings Department, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

March 1997
CERLIB 3rd Winter Research Conferences, Les Arcs 1800, France, March 9–14, 1997. Proteins and Free Radicals, from Radical Enzymes to Damages; Oxidative Effects of Radiations on Biomolecules and Cells; Oxidative Stress and Apoptosis. Information: Arlette Alcaraz, Laboratoire de Biochimie C, CHU Grenoble BP 217, 38043 Grenoble Cédex 9, France (Phone: 33 76 76 57 54; Fax: 33 76 76 56 64; E-mail: CERLIB@ujf-grenoble.fr).

April 1997
IV. International Dermatology Symposion Berlin, Sebaceous Gland and Its Disorders, Basic and Clinical Research, Clinical Entities and Treatment, April 11–13, 1997, University Medical Center Benjamin Franklin. Information: Docent Dr. Ch. C. Zouboulis, Department of Dermatology, University Medical Center Benjamin Franklin, The Free University of Berlin, Hindenburgdamm 20, D-12200 Berlin, Germany (Phone: 49-30-8445-2808; Fax: 49-30-8445-4262).

June 1997
The 19th Nordic Lipid Symposium, Ronneby, Sweden, June 15–18, 1997. Information: Georg Lambertsen, Secretary, Lipidforum, Breistølen 1, N-5035, Bergen, Norway (Phone: 47 55 + 32 89 56; Fax: 47 55 + 32 57 24).

July–August 1997
Fourth International Congress on Essential Fatty Acids and Eicosanoids, July 27–August 1, 1997, Edinburgh, Scotland. Information: Secretariat, c/o Meeting Makers, 50 George St., Glasgow G1 1QE, Scotland, United Kingdom.

Acyl-CoA Binding Proteins: Multiplicity and Function

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ABSTRACT: The physiological role of long-chain fatty acyl-CoA is thought to be primarily in intermediary metabolism of fatty acids. However, recent data show that nM to μM levels of these lipophilic molecules are potent regulators of cell functions *in vitro*. Although long-chain fatty acyl-CoA are present at several hundred μM concentration in the cell, very little long-chain fatty acyl-CoA actually exists as free or unbound molecules, but rather is bound with high affinity to membrane lipids and/or proteins. Recently, there is growing awareness that cytosol contains nonenzymatic proteins also capable of binding long-chain fatty acyl-CoA with high affinity. Although the identity of the cytosolic long-chain fatty acyl-CoA binding protein(s) has been the subject of some controversy, there is growing evidence that several diverse nonenzymatic cytosolic proteins will bind long-chain fatty acyl-CoA. Not only does acyl-CoA binding protein specifically bind medium and long-chain fatty acyl-CoA (LCFA-CoA), but ubiquitous proteins with multiple ligand specificities such as the fatty acid binding proteins and sterol carrier protein-2 also bind LCFA-CoA with high affinity. The potential of these acyl-CoA binding proteins to influence the level of free LCFA-CoA and thereby the amount of LCFA-CoA bound to regulatory sites in proteins and enzymes is only now being examined in detail. The purpose of this article is to explore the identity, nature, function, and pathobiology of these fascinating newly discovered long-chain fatty acyl-CoA binding proteins. The relative contributions of these three different protein families to LCFA-CoA utilization and/or regulation of cellular activities are the focus of new directions in this field.
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HISTORICAL PERSPECTIVE

Long-chain fatty acyl-CoA (LCFA-CoA) are the CoA thioesters of fatty acids (Fig. 1A) and represent the "activated state" of fatty acids necessary for metabolism. Early studies of CoA derivatives of long-chain fatty acids focused on their function(s) as intermediates in fatty acid metabolism. More recent data suggesting potential roles of LCFA-CoA in cellular signalling and in gene regulation have rekindled interest in these established lipidic molecules. Despite this impor-

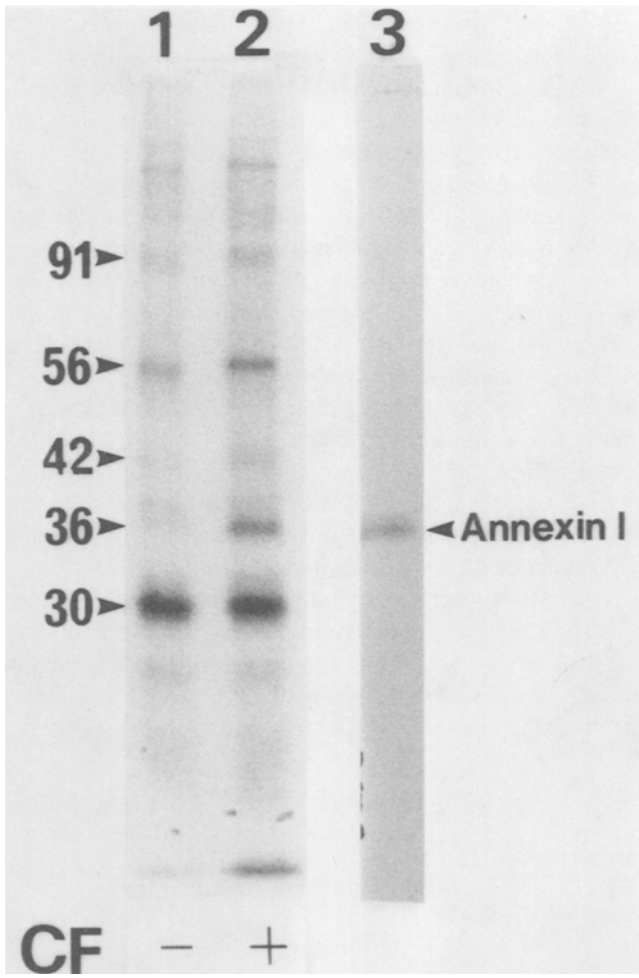


FIG. 1. Structure of free (panel A) and liver fatty acid binding protein bound (panel B) oleoyl CoA. These structures were prepared by Silicon Graphics (Mountain View, CA) computer modeling as described elsewhere (Ref. 127).

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Abbreviations: ACBP, acyl-CoA binding protein; ACC, acetyl-CoA carboxylase; ALBP, adipocyte lipid binding protein; ANT, adenine nucleotide translocase; B-FABP, brain fatty acid binding protein; DBI, diazepam binding inhibitor; GABA, gamma aminorbutyric acid; H-FABP, heart fatty acid binding protein; I-FABP, intestinal fatty acid binding protein; L-FABP, liver fatty acid binding protein; LCFA-CoA, long-chain fatty acyl-CoA; NBD-stearate, 12-*N*-methyl-(7-nitrobenz-2-oxa-1,3-diazol)amino stearate; NMR, nuclear magnetic resonance; ODN, octadecaneuropeptide; PBR, peripheral benzodiazepine receptors; SCP-2, sterol carrier protein-2; tricine, *N*-[tris-(hydroxymethyl)methyl]glycine; TTN, triacontatetraneuropeptide.

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tance, very little is known regarding the intracellular trafficking, targeting, and presentation of LCFA-CoA to enzymes and DNA regulatory binding proteins. LCFA-CoA may move through the cytoplasm either spontaneously or by protein mediated intracellular movement. The latter mechanism is particularly appealing since it offers a means of specific intracellular targeting for LCFA-CoA. Nearly two decades ago it was reported that the cytosol contained LCFA-CoA binding activity, subsequently identified as fatty acid binding proteins (FABP) (1–5). However, the identity of FABP as being responsible for cytosolic LCFA-CoA binding has recently been questioned (6–8). What then is(are) the cytosolic LCFA-CoA binding protein(s) and what is(are) their physiological function(s)? Without attempting to be exhaustive, the remainder of this article is focused on examining the identity, tissue distribution, binding characteristics, function, molecular biology, structure, regulation, and pathobiology of the cytosolic, nonenzymatic, LCFA-CoA binding proteins.

THE LIGAND: LCFA-CoA

Tissue content and distribution of LCFA-CoA. LCFA-CoA are present in the cell in high amounts. Although the majority of studies quantitating LCFA-CoA have focused on liver, heart, and lung, significant amounts also have been found in brain, kidney, muscle, and adipose tissues (Table 1). The data have been difficult to compare, largely because LCFA-CoA levels have been reported in many different units. For example, liver concentration of LCFA-CoA has been expressed on the basis of tissue wet weight (15–83 nmol/g wet weight; with most values centered near 40 nmol/g wet weight), dry weight (94–219 nmol/per g dry weight), protein (108–248 nmol/g cell protein), and as μM (110–152 μM in liver cytosol). Heart LCFA-CoA levels are in the same range as those of liver (10–70 nmol/g wet weight; with most values centered near 50 nmol/g wet weight). Tissues such as lung, brain, and kidney have intermediate levels near 24–31 nmol/g wet weight. In contrast, muscle and adipose LCFA-CoA concentrations are up to 5- to 35-fold lower than liver or heart (Table 1). The high levels of LCFA-CoA in liver and heart are consistent with the role of these tissues in LCFA-CoA metabolism: glyceride and cholesteryl ester synthesis in the liver and fatty acid oxidation in the heart. Thus, it seems surprising that adipose tissue has extremely low levels of LCFA-CoA. This suggests that other factors (e.g., LCFA-CoA thiolase, acyl transferase enzyme activity, LCFA-CoA binding proteins) must play a role in determining tissue LCFA-CoA content.

TABLE 1
Tissue Long-Chain Fatty Acyl-CoA Content

Tissue	Content	Units	Reference
Liver	15–83	nmol/g wet weight	15,16,156,198–200,204,208
	94–219	nmol/g/dry weight	201
	108–248	nmol/g protein	39
	110–152	μM	202
Heart	10–70	nmol/g wet weight	17,38,203,204,208
	59	nmol/g/dry weight	203
Lung	6–31	nmol/mg protein	11,205
Brain	23–27	nmol/g wet weight	204,206
Kidney	24	nmol/g wet weight	204
Muscle	4–10	nmol/g wet weight	38,204
Adipose	2	nmol/g wet weight	207

Compartmentalization of LCFA-CoA. In most tissues LCFA-CoA is generally present in highest concentration in mitochondria and microsomes (Table 2). This would be expected due to the presence of enzymes involved in β-oxidation and fatty acid synthesis in these locations. In contrast, cytosolic LCFA-CoA levels may be relatively low or undetectable. For example, two studies reported that LCFA-CoA were not detected in cytosol of rat liver and rat heart (9,10). In contrast, LCFA-CoA content of rat lung homogenate, microsomes, and cytosol was 16.8 ± 7.6, 14.4 ± 7.9, and 2.3 ± 0.3 nmol/mg protein, respectively (11). Furthermore, assuming that about 20% of cellular protein is cytosolic, then only 2.7% of lung LCFA-CoA is cytosolic. In contrast, a similar calculation for lung alveolar type II cells (11) reveals that in these cells about 21% of cellular LCFA-CoA is cytosolic. Other reports also indicate that cytosolic LCFA-CoA content may be substantial. For example, if one assumes that cytosol represents about 20% of cell protein, two reports (12,13) indicate that about 17 and 60% of LCFA-CoA may be cytosolic in rat liver and heart, respectively (Table 2). The reasons for the discrepancies between these reports on the low or absence vs. significant levels of LCFA-CoA in liver and heart cytosol are not known.

The subcellular distribution of LCFA-CoA can be modulated by the oxygenation state of the tissue. For example, hypoxia resulted in a 4.5-fold increase in LCFA-CoA levels of rat heart cytosol (13). Concomitantly, hypoxia increased

TABLE 2
Subcellular Distribution of Long-Chain Fatty Acyl-CoA in Rat Tissues

Tissue	Homogenate	Microsomes	Mitochondria	Cytosol	Reference
Lung[a]	16.8 ± 7.6	14.4 ± 7.9	NA	2.3 ± 0.3	11
Alveolar Type II cells[a]	31.3 ± 10.8	92.7 ± 7.6	NA	33.2 ± 6.3	11
Liver[b]	51.5 ± 2.6	NA	7.5 ± 2.6	43.8 ± 2.2	12
Liver[a]	NA	0.11, 0.10	0.1, 0.5	NA	9
Heart[a,b]	14.0 ± 0.8[b]	NA	0.15 ± 0.01[a]	NA	10
Heart[a,b]	30 ± 10[b]	NA	0.26 ± 0.02[c]	18 ± 4[c]	13

[a]nmol/mg protein. [b]nmol/g wet weight. [c]μM. NA, not assessed.

LCFA-CoA levels 6- to 7-fold in rat heart and liver mitochondria (10).

Effect of drugs and disease on tissue LCFA-CoA. The level of LCFA-CoA in the cell is not a constant parameter but is highly dependent on a number of other factors. LCFA-CoA levels are modulated over a 20-fold range by nutrition, drugs, oxygenation, and endocrine status, resulting in increased LCFA-CoA levels severalfold in liver and/or heart (Table 3). For example, administration of long-chain fatty acids such as palmitate and drugs such as peroxisome proliferators doubled liver LCFA-CoA content (14). Starvation increased liver LCFA-CoA content nearly 4-fold (15–17). Furthermore, ischemia and hypoxia increased heart LCFA-CoA levels up to 1.6- to 3.1-fold (10). In contrast, valproate induced hepatotoxicity decreased liver LCFA-CoA content nearly 5-fold (18) (Table 3).

Function of LCFA-CoA. LCFA-CoA have long been recognized as being important intermediates in the *de novo* biosynthesis of fatty acids (endoplasmic reticulum) as well as fatty acid oxidation (mitochondria and peroxisome), membrane biogenesis (phospholipids), and lipid storage (triglycerides and cholesteryl esters) of exogenously derived fatty acids in the cell (Table 4). Equally important, LCFA-CoA feedback inhibit several enzymes in fatty acid synthesis and metabolism [e.g., fatty acid synthetase and acyl CoA synthetase (19–23)]. In view of the importance of these lipids in atherosclerosis, diabetes, and other lipid disorders alone, LCFA-CoA occupy an important place in biology.

Less well understood is the role of fatty acyl-CoA as regulatory molecules in signaling pathways. For example, LCFA-CoA modulate neutrophil superoxide generation (24), intracellular Ca^{++} (25), glucose-stimulated insulin release (26), protein acylation (27–29), and protein phosphorylation (30,31) (Table 4). Equally important, exciting recent reports from bacterial systems indicate that LCFA-CoA have additional roles in regulating gene transcription (33,34) (Table 4).

Mechanism of LCFA-CoA modulation of protein function. Despite the demonstrated impacts on regulation of cellular function *in vitro*, there has been some concern that regulatory effects of LCFA-CoA may be based on nonspecific and artifactual detergent activity of these highly surface-active molecules (35). Some support for this concern comes from early studies that used dye-binding techniques to measure the critical micellar concentration of LCFA-CoA, near 3–4 μM (reviewed in Ref. 35). If this technique correctly measures the LCFA-CoA critical micellar concentration, then one must conclude that in the 100–150 μM range of cellular LCFA-CoA concentrations, these molecules could form micellar structures. Such micelles could act as potent detergents to disrupt the structure of membrane lipids and/or to directly solubilize/disrupt protein structure (reviewed in Ref. 35). This would elicit potent deleterious effects on membrane-bound or soluble proteins and enzymatic processes (reviewed in Ref. 35). However, three sets of evidence are inconsistent with the above conclusion.

First, the dye-binding techniques used to measure the critical micellar concentrations for LCFA-CoA yield critical micellar concentrations that are several orders of magnitude too low. A wide variety of more recent studies with fluorescence, spin-label, ultrastructural, and gel permeation techniques showed that the critical micelle concentration of LCFA-CoA was two orders of magnitude higher, between 60–200 μM (reviewed in Ref. 35), than obtained with the dye-binding technique. Thus, under normal physiological conditions, adipose, muscle, kidney, brain, and lung tissue levels of total cellular LCFA-CoA (about 2–70 μM) are considerably less than the LCFA-CoA micellar concentration range (Table 1). Although the proportion of cytosolic LCFA-CoA in most of these tis-

TABLE 3
Modulation of Tissue Long-Chain Fatty Acyl-CoA

Parameter	Change	Reference
	Liver	
Starvation	2.2- to 3.7-fold ↑	15–17
Diabetes	1.2- to 1.9-fold ↑	198
Hepatotoxicity	5.0-fold ↓	18
Peroxisome proliferation	1.5- to 1.8-fold ↑	14
Palmitic acid	1.9-fold ↑	14
	Heart	
Ischemia	1.6- to 3.1-fold ↑	10
Hypoxia	1.7-fold ↑	10

TABLE 4
Functional Processes Regulated by Fatty Acyl-CoA

Process	Protein regulated	Reference
	Metabolism	
Fatty acid synthesis	Acetyl-CoA carboxylase	40
	Fatty acid synthetase	19,20
Fatty acid oxidation	Thiolase I and II	35
	Acyl-CoA synthase	21,22
Glycolysis	Glucokinase	209
Citric acid cycle	Citrate synthase	210
Ketogenesis	Citrate synthase	210,211
Energy metabolism	Malate dehydrogenase	212
	ATP/antiporter	22,213
Drug metabolism	Chloramphenicol acetyl transferase	214,215
	Signalling	
Neutrophil signalling	O_2-generation	24
Ca^{++}	Intracellular Ca^{++} set point	25,32
Insulin release	Glucose stimulated insulin release	26
Protein acylation	Myristoyl CoA/protein-*N*-myristoyl-transferase	27,28
	Palmitoyl CoA:protein-*N*-palmitoyl-transferase	29
Protein phosphorylation	Protein kinase	30,31
	Gene transcription	
Transcription	Transcription factor Fad R	33,34

sues is unknown, lung has only about 3% of its LCFA-CoA in the cytosol. Likewise, although liver or heart LCFA-CoA concentrations are about 70–210 μM (Table 1), generally less than one-fifth of this LCFA-CoA is cytosolic (Table 2). These considerations indicate that cytosolic LCFA-CoA concentrations in a variety of tissues are in the range of 0 to 42 μM, clearly below the critical micellar concentration of LCFA-CoA.

More importantly, under a variety of conditions [starvation, diabetes, peroxisome proliferators, fatty acid feeding, ischemia, hypoxia (Table 3); pathological conditions (see last section of this article)], liver and heart LCFA-CoA levels are as much as 4-fold higher and well above the range of LCFA-CoA critical micellar concentration in most tissues reported. Unless substantial quantities of LCFA-CoA are specifically bound by cytosolic LCFA-CoA binding proteins or nonspecifically bound by membrane lipids, these levels of LCFA-CoA would be toxic due to nonspecific binding to and subsequent inhibition of enzymes vital to cell function.

In addition, the intracellular level of free (not bound to protein or membranes) LCFA-CoA levels in the cell is much lower because of intracellular binding by membrane lipids and binding to cytosolic long chain acyl CoA binding proteins. LCFA-CoA will partition between membrane phospholipids and cytosolic proteins, depending on their respective relative affinities and capacities for LCFA-CoA binding. Since phospholipid membranes can bind up to 15 mol% LCFA-CoA without disruption, they have a large capacity for binding the levels of LCFA-CoA found in cells (36,37). In addition, liver cytosolic lipid binding proteins bind about 450 nmole/g cytosolic protein. Liver LCFA-CoA levels are 108–248 nmole/g total protein in fed and fasted rats (39). If one takes 20% of cellular LCFA-CoA to be cytosolic and assumes that cytosolic protein accounts for about 20% of cellular protein, one can estimate that about 5–10% of the available cytosolic protein LCFA-CoA binding sites are normally occupied by LCFA-CoA. This estimate is in agreement with one recent study reporting the LCFA-CoA pool sizes in lung type II cells (11): whole cells, 31 ± 11; microsomes, 93 ± 8; cytosol, 33 ± 6 nmol/mg protein (11). Thus, even if the cytosolic LCFA-CoA content increases 4–5-fold as in ischemia (Table 3), the available cytosolic protein LCFA-CoA binding sites would still not be saturated with LCFA-CoA.

The remaining noncytosolic LCFA-CoA appears bound to membrane lipids and/or proteins. The LCFA-CoA acyl chain composition of cytosolic and microsomal fractions of the lung type II cells was identical, with 16:0 (49–54%), 18:0 (20–21%), and 18:1 (14–16%) acyl CoA predominating (11). This would suggest that the membranes and cytosolic LCFA-CoA binding proteins (as a group) display similar affinity profiles for a variety of LCFA-CoA. However, other data obtained with individual purified LCFA-CoA (see below) indicate that significant differences in LCFA-CoA acyl chain preferences may exist.

Since the membrane lipids provide a huge reservoir of additional nonspecific LCFA-CoA binding sites, it is likely that there exists very little cytosolic free-unbound LCFA-CoA. The above observations certainly are consistent with this prediction. The following calculation provides an estimate of the free-unbound cytosolic LCFA-CoA. Since cytosolic proteins represent about 20% of total cell protein, the above data may be used to estimate that 74% of LCFA-CoA is bound nonspecifically to membranes as compared to 20% in cytosol [free + cytosolic protein bound]. Because liver has 108–248 nmol LCFA-CoA/g protein and if 20% is cytosolic, then 22–50 nmol LCFA-CoA/g cytosolic protein is present in cytosol. Cytosolic long-chain fatty acyl-CoA binding proteins specifically bind up to 446 nmol LCFA-CoA/g cytosolic protein in liver, therefore exceeding the LCFA-CoA concentration by 9- to 20-fold. Since the K_d of the LCFA-CoA binding proteins are in the 0.08–10 μM range (see Table 8 below), cytosolic LCFA-CoA are probably 95% bound to the LCFA-CoA binding proteins. The level of cytosolic free-unbound LCFA-CoA is estimated to be in the range 1–4 μM, based on the above calculations. However, depending on the tissue type, this level of free-unbound LCFA-CoA would depend on the type, quantity, and relative affinities of the cytosolic LCFA-CoA protein(s) present.

Finally, the level of LCFA-CoA necessary to modulate the function of enzymes and functions *in vitro* (Table 4) are generally 0.5–10 μM. Clearly, these levels are not only below the critical micellar range of 60–200 μM, but they are in the estimated range of cytosolic free-unbound LCFA-CoA near 1–4 μM (see above). It should be noted that enzymes such as mitochondrial ATP/ADP translocase (ANT) and acetyl CoA carboxylase (ACC) are both inhibited by nanomolar concentrations of fatty acyl CoA (22,23,40). Likewise, inhibition of transcription factor proteins (e.g., FadR) occurs at low concentration, 12 nM (33,34). Furthermore, the effects of LCFA-CoA appear to be specific since they are not observed upon addition of short-chain acyl-CoA (33,34). Thus, not only the cytosolic levels of free-unbound LCFA-CoA, but also their subcellular distribution (peroxisomes, mitochondria, nucleus, cytosol), are important in the regulation of cellular function. In summary, the levels of free-unbound LCFA-CoA eliciting regulatory effects are 3–4 orders of magnitude lower than the critical micellar concentration. They are, however, in the range potentially influenced by the cytosolic LCFA-CoA binding proteins.

IDENTITY AND TISSUE DISTRIBUTION OF MAMMALIAN LCFA-CoA BINDING PROTEINS

Although albumin binds LCFA-CoA and is found in liver (37), it is primarily an extracellular serum protein and will not be considered further here. At least three different types of cytosolic LCFA-CoA binding proteins have been discovered: FABP, acyl CoA binding protein (ACBP), and sterol carrier protein-2 (SCP-2) (Table 5).

Cytosolic FABP. Perhaps the first intracellular proteins recognized as fatty acyl CoA binding proteins were the cytosolic FABP from liver and heart (2–5,41,42) (Table 5). Seventeen

TABLE 5
Cytosolic Fatty Acyl-CoA Binding Protein Families

Family	Protein	Acronym[a]
Fatty acid binding protein	Liver fatty acid binding protein	L-FABP
	Heart fatty acid binding protein	H-FABP
	Intestinal fatty acid binding protein	I-FABP
	Brain fatty acid binding protein	B-FABP
	Adipocyte lipid binding protein	ALBP
Acyl-CoA binding protein	Acyl-CoA binding protein	ACBP
Sterol carrier protein-2	Sterol carrier protein-2	SCP-2
	Nonspecific lipid transfer protein	nsLTP
	Sterol carrier protein-x	SCP-x

[a]FABP, fatty acid binding protein (L-, liver; H-, heart; I, intestinal; B, brain); ALBP, adipocyte lipid binding protein; ACBP, acyl-CoA binding protein; SCP-2, sterol carrier protein-2 (13-2 kDa); SCP-x, sterol carrier protein-x (58 kDa).

members of this intracellular FABP family have been identified (42,43). The FABP are 14–16 kDa clam-shaped proteins present in high concentration in the cell (2–5% of cytosolic protein), and their properties are reviewed in References 42–45. Within the cell, FABP are primarily cytosolic, with highest concentrations near the endoplasmic reticulum and lower levels localized in mitochondria and nucleus (46,47). Although the individual FABP appear unique in regard to sequence and antigenic specificity, their ligand specificities are generally broad and overlapping and include fatty acids, lysophosphatidic acid, prostaglandins, cholesterol (in some cases), bile acids, azo dyes, carcinogens, etc. (reviewed in Refs. 44,45,48). Twenty years ago, the first FABP shown to bind LCFA-CoA was the native L-FABP isolated from rat liver (1,2,4). This observation was later followed by reports that native heart FABP (H-FABP) (3,5,44), native adipocyte lipid binding protein (ADLP) (42), recombinant intestinal-FABP (I-FABP; Jolly, C., Hubbell, T., Behnke, W.D., and Schroeder, F., submitted for publication), brain recombinant brain-fatty acid binding protein (B-FABP) (49), and brain recombinant H-FABP (49) also bound LCFA-CoA (Table 5). However, the results from LCFA-CoA binding studies obtained with native FABP isolated from liver, heart, and adipose tissues were recently questioned (7) when a new LCFA-CoA binding protein, acyl-CoA binding protein (ACBP), was isolated from the liver (6). It was suggested that early preparations of FABP isolated from native tissues did not resolve low molecular weight proteins such as ACBP. Thus, it seemed possible that ACBP co-purifying with the native FABP might account for the LCFA-CoA binding properties of these preparations (7). This dilemma was resolved by the use of recombinant liver-FABP (L-FABP) (41). Bacteria do not contain acyl-CoA binding protein (ACBP), and therefore this mammalian protein cannot be a cross contaminant in recombinant L-FABP preparations. Furthermore, when the recombinant L-FABP was subjected to Tricine (Fisher, Pittsburgh, PA) SDS–polyacrylamide gel electrophoresis capable of resolving 10 kDa ACBP from 14 kDa L-FABP, no bands were detected at 10 kDa. Finally, several assays including a modified Lipidex 1000 (Sigma, St. Louis, MO) assay (41), a fluorescent *cis*-parinaroyl CoA binding assay (41), *cis*- and *trans*-parinaroyl CoA lifetime, limiting anisotropy, and rotations (Frolov, A., and Schroeder, F., submitted for publication), and L-FABP intrinsic tyrosine fluorescence (Jolly, C.A., Hubbell, T., Behnke, W.D., and Schroeder, F., submitted for publication) clearly demonstrated LCFA-CoA interaction with recombinant L-FABP.

ACBP. The second LCFA-CoA binding protein family has been the subject of considerable interest and controversy. Thus far, only one member of this family has been reported, but with two very different designations and/or potential functions. It was first discovered in 1983 in the brain and designated as a diazepam binding inhibitor (DBI) protein (50). Initially DBI was thought to function as a potential neurotransmitter which displaced benzodiazepines from GABA receptors in the central nervous system (50,51). It is identical in amino acid and cDNA sequence to ACBP, isolated from bovine liver three years later (6,52). Although this LCFA-CoA binding protein is variously designated as ACBP, DBI, or ACBP/DBI for simplicity's sake, only the ACBP designation will be used in this review. ACBP is a highly conserved protein found in a wide range of animals including yeasts, insects (tobacco hornworm, Drosophila), frogs, trout, tortoise, birds, and mammals (rat, mouse, rabbit, dog, pig, cow, and human) as well as plants (6,19,51,53–66). ACBP has been identified in all mammalian tissues tested to date (19,51,53, 67–77).

ACBP is primarily a cytosolic protein that appears localized in highest concentrations in specialized cells. Immunohistochemistry has been used to resolve ACBP localization in tissues. ACBP is immunodetected primarily in cells having secretory function such as hepatocytes, steroid producing cells of the adrenal cortex and testis, cells in islets of the pancreas, tubular epithelial cells of the kidney, small intestinal epithelial cells, bronchiolar epithelial cells, etc. (70,71). In the brain, ACBP has been localized primarily to ependymal and glial cells, with low levels in neurons in some species (78–81). However, within the neuron ACBP appears to be

TABLE 6
Tissue Levels of Acyl-CoA Binding Protein (ACBP)

Tissue	Rabbit[a] (nmol/g wet weight)	Bovine[b] (nmol/mg cytosol protein)	Rat[c] (nmol/mg cytosol protein)	Human[a] (nmol/g wet weight)
Liver	0.15	0.33 (15)[d]	0.64[e] (28)[d]	—
Intestine mucosa	—	0.15	0.57	—
Stomach	0.13	—	—	—
Colon	0.11	—	—	—
Adipose	—	0.18	—	—
Mammary	—	0.24	—	—
Kidney	0.34	0.14	0.13	—
Heart	0.07	0.12 (4)[d]	0.05 (2)[d]	—
Brain	0.38	0.12 (1.15)[f]	—	1.57
Muscle	—	0.06	0.04	—
Erythrocyte	—	—	1.21	—
Plasma	ND	—	0.01	—

[a]Reference 51. See Table 5 for abbreviation. [b]Reference 68. [c]Reference 19. [d]nmol/g wet weight (Ref. 146). [e]408 nmol/g liver protein (Ref. 19). [f]nmol/g wet weight (Ref. 51).

concentrated in the synaptosomal nerve ending (82). Mouse brain synaptosomal cytosol ACBP concentration is high at 3.1 μg/mg (0.31 nmol/mg) cytosolic protein (82). This level observed in neuronal nerve endings (synaptosomes) is as high as that reported for liver or intestine, organs with highest reported levels of ACBP (Table 6). These observations with neurons and nerve endings suggest that, although ACBP is primarily cytosolic, its distribution within the cytosol may not necessarily be uniform.

Sterol carrier protein-2 (SCP-2). The third long-chain fatty acyl-CoA binding protein, SCP-2, is the newest member of the LCFA-CoA binding proteins (Frolov, A., Cho, T.-Y., Billheimer, J.T., and Schroeder, F., submitted for publication) (Table 5). Tissue levels of SCP-2 are in the range of 0.01 to 0.10% of cytosolic protein (83–85). SCP-2 is alternately known as nonspecific lipid transfer protein, nsLTP, a designation based on its broad ligand specificity for phospholipids (85), glycolipids (84), cholesterol (86), fatty acids (87), and fatty acyl-CoA (see below) (Frolov, A., Cho, T.-Y., Billheimer, J.T., and Schroeder, F., submitted for publication). Two immunodetectable SCP-2 forms have been demonstrated with the 13.2 kDa SCP-2 generally being present in 10-fold greater amount than the 58 kDa SCP-x. More importantly, the subcellular distribution of these SCP-2 forms differs dramatically. The 13.2 kDa SCP-2 protein is present in cytosol, mitochondria, peroxisomes, and near endoplasmic reticulum, while the 58 kDa SCP-x form is localized exclusively in peroxisomes (88).

Relative concentrations of FABP, acyl-CoA binding protein, and SCP-2 within tissues. The data provided in the above sections allow direct comparisons of the relative concentrations of the three LCFA-CoA binding protein families in a variety of tissues. For example, L-FABP levels are generally in the range of 3–5% of cytosol protein (2.2–3.6 nmol/mg cytosol protein) (3,44). Liver ACBP levels are found in the range from 0.06 to 0.64 nmol/mg cytosol protein (on average about 0.33 nmol/mg cytosol protein) (19,68). Liver levels of SCP-2 are about 0.10% of cytosolic protein (0.07 nmol/mg cytosol protein) (83–85). Thus, the overall pattern of the relative molar ratio of these proteins (L-FABP/ACBP/SCP-2) reported in liver cytosol appears to be approximately 40:5:1. These ratios are also reflected in some tissues high in all three proteins, i.e., intestine, but not in many other tissues such as brain. In mouse brain, the relative molar ratio of FABP/ACBP/SCP-2 calculated from reported values (49,82) is approximately 3:1:1, very different from that observed in liver. Taken together with their somewhat different subcellular distributions (see above), this would suggest that the relative importance of FABP, ACBP, and SCP-2 in LCFA-CoA metabolism and/or regulation of cellular function may be highly dependent on the specific tissue and cell type within that tissue.

MOLECULAR BIOLOGY OF CYTOSOLIC LCFA-CoA BINDING PROTEINS

As shown in Table 5, the cytosolic LCFA-CoA binding proteins belong to at least three different families. Within a specific family there is considerable amino acid and cDNA sequence homology/identity. In contrast, there is very little if any identity/homology in amino acid or cDNA sequence between FABP (reviewed in Refs. 44,45), acyl-CoA binding proteins (reviewed in Refs. 19,54–60), and SCP-2 (reviewed in Refs. 90,91).

Cytosolic FABP. The molecular biology of the FABP family has been extensively reviewed previously (42–45) and will not be reiterated here. Suffice it to say that nearly half of the reported seventeen members of this family have been cloned and sequenced with their amino acid sequence homology within a species ranging from 18–67% (reviewed in Ref. 43). Sequence homology of a given FABP among several species is, of course, considerably greater. Exons III and IV are thought to be related to the differences in ligand specificities between members of the protein family or to targeting of specific enzymes or membranes with which they interact.

ACBP. In contrast to the FABP family, a single gene appears to code for a single protein, ACBP, which appears to be present in all tissues tested. Comparisons of the ACBP amino acid sequence of different organisms shows high sequence homology with most observed differences represented by conservative changes (58,92). The ACBP gene, comprised of four exons and three introns, has been cloned and characterized in the rat (60,65), human (93), duck (58), and yeast (59). The rat ACBP has one expressed gene, localized on chromosome 2, and four processed pseudogenes. ACBP gene transcription is initiated at two major and multiple minor initiation sites. The promoter region displays several potential tissue-specific *cis*-acting elements that may regulate the level of tissue-dependent ACBP expression. Alternative RNA splicing in the ACBP gene is not observed. The ACBP gene shows characteristics of a typical housekeeping gene (60,65).

SCP-2. The SCP-2 proteins of rat, mouse, human, and chicken have been cloned and sequenced (reviewed in Refs. 84,94,95). The chicken SCP-2 is 88% identical to its mammalian homologs, consistent with a high degree of evolutionary conservation (94). SCP-2 cloned from a mouse brain library is 99.9% identical in cDNA and amino acid sequence to SCP-2 cloned from a liver cDNA library, indicating that a single gene encodes for SCP-2 (82). The SCP-2 gene is located on human chromosome 1p32 and consists of 15 exons and 15 introns (95). Multiple transcription start sites have been identified. The presence of two unique initiation sites and a leader sequence (post-translationally cleaved) in the SCP-2 gene results in the expression of two proteins of different molecular weight, 15 kDa (pro-SCP-2) and 58 kDa (SCP-x) (90,91). Alternative RNA splicing in the SCP-2 gene has been suggested to give rise to a 29 kDa SCP-2 (96). This suggestion appears consistent with Western blots of liver cytosol showing cross reactivity of anti-SCP-2 with a protein near 29 kDa. However, work from our laboratory in which the 29 kDa protein was isolated from rat liver indicated no similarity to SCP-2 or the putative 29 kDa SCP-2 suggested to arise by alternative RNA splicing (Pu, L., and Schroeder, F., manuscript in preparation). Likewise, higher molecular weight proteins cross reactive with anti-SCP-2 have also been revealed by Western blots of yeast (97), but these proteins either did not function in lipid transfer (97) or, when isolated and/or cloned, were unrelated to SCP-2 or the putative 29 kDa SCP-2 suggested to arise by alternative RNA splicing (Tahotna, D., Ossendorp, B., Schroeder, F., Daum, G., and Hapala, I., manuscript in preparation). Thus, it would seem that the SCP-2 gene codes only for two transcripts transcribed as 58 kDa SCP-x and 15 kDa pro-SCP-2. The 15 kDa pro-SCP-2 is rarely detected in tissues because its presequence is rapidly post-translationally cleaved to yield the 13.2 kDa SCP-2 protein (90,91). The 58 kDa SCP-x and 13.2 kDa SCP-2 proteins share a common 13.2 kDa C-terminus. The 13.2 kDa SCP-2 is reported localized to cytosol, mitochondria, peroxisomes, and near endoplasmic reticulum while the 58 kDa SCP-x is found exclusively in peroxisomes (88).

REGULATION OF THE LCFA-CoA BINDING PROTEINS

Cytosolic FABP. The influence of physiological conditions, nutrition, development, sex, diurnal rhythm, and drugs on FABP expression has been reviewed previously and will not be reiterated here (44,98–104). Suffice it to say that under these conditions, FABP levels range from up to 6-fold decreases to 3-fold increases, depending on the type of FABP and tissue examined.

ACBP. The influence of nutrition on ACBP levels has been examined in rat liver, kidney, and heart. In one study, fasting for 24 to 48 h resulted in elevation of long-chain acyl-CoA esters, decreased liver ACBP mRNA levels, and no significant change in ACBP protein levels measured by an ELISA assay (105). In a second study, ACBP tissue levels, especially in liver, responded acutely to changes in diet (106). Fasting for 24 to 48 h resulted in decreased liver ACBP levels (from 66.5 ± 13.1 to 39.5 ± 6.2 pg/ng DNA) but had no effect on kidney and heart levels. Feeding of a high-fat diet resulted in an approximately 1.5- to 2-fold increase in liver ACBP levels by 48 h and levels remained elevated as long as the high-fat diet was fed. Kidney and heart showed a mild increase in ACBP levels after prolonged feeding of the high-fat diet (by 7 and 28 d, respectively) (106).

Like FABP, ACBP is regulated by peroxisome proliferators. Several peroxisome proliferators have been shown to induce increased expression of rat hepatic ACBP RNA and protein levels approximately 1.5- to 2-fold within 24 to 48 h of exposure *in vitro* and ACBP protein levels by seven days *in vivo* (105,107). *In vivo*, ACBP mRNA and protein levels increased in a time- and dose-dependent fashion. ACBP mRNA increased by 24 h post exposure and protein levels increased 1.5- to 1.7-fold by 3–14 d of exposure (14). The induction of long-chain acyl-CoA hydrolase activity increased in parallel to the ACBP increase and may be due to peroxisome proliferator induced sustained accumulation of total long-chain acyl CoA esters (14).

L-FABP and I-FABP stimulate the formation of glycerides from LCFA-CoA, while neither L-FABP nor I-FABP (intestinal FABP) stimulates formation of LCFA-CoA by LCFA-CoA synthase (41; Jolly, C.A., Hubbell, T., Behnke, W.D., and Schroeder, F., submitted for publication). I-FABP expression in transfected CaCo-2 cells decreased L-FABP expression (89). Therefore it seems possible that cellular L-FABP and/or I-FABP levels could modulate the expression of ACBP. The effect of L-FABP and I-FABP levels on expression of ACBP was examined in transfected L-cell fibroblasts. L-cells transfected with either L-FABP or I-FABP differentially affect the uptake of fatty acids and the synthesis of phospholipids vs. triglycerides (108). However, neither L-FABP nor I-FABP expression affected the expression of ACBP in L-cells (Fig. 2): untransfected L-cells, LM-cells (derived from L-cells), transfected L-cells with 0.4% of cytosol protein as L-FABP, and transfected L-cells expressing about 0.35% of cytosol protein as I-FABP were 0.83 ± 0.15, 0.72 ±

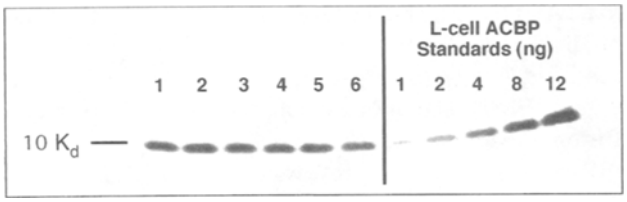


FIG. 2. Acyl-CoA binding protein (ACBP) levels in transfected L-cell fibroblasts expressing other long-chain fatty acyl-CoA binding proteins. The derivation of liver fatty acid binding protein (L-FABP) (151,229), intestinal-FABP (I-FABP) (108), and sterol carrier protein-2 (SCP-2) (126) expressing cell lines, SDS–polyacrylamide gel electrophoresis, and Western blotting procedures are described therein. Immunoblotting was performed with rabbit anti-L-cell ACBP polyclonal primary antibody; biotinylated goat anti-rabbit IgG as secondary antibody; avidin biotin-alkaline phosphatase, and visualization with Vector black substrate (Vector, Burlingame, CA). Blots were photographed with an IS-500 computerized gel documentation system (Alpha-Innotech, San Leandro, CA). From each image, protein bands of cell samples in each lane were quantitated by comparing to known amounts of ACBP protein standards loaded on the same blot using the public domain program, NIH Image V.I.G. (written by Wayne Rasband and is available by anonymous FTP from zippy.nimh.nih.gov.) on a Power MacIntosh 8100/80AV computer (Apple Corp., Cupertino, CA). ACBP protein was expressed as ng/μg sample. The amount of ACBP in the six cell lines analyzed was not significantly different ($P > 0.05$) and ranged from 0.50 to 0.83 ng/μg protein. A representative immunoblot is shown. Lanes 1–6 were loaded with 10 μg protein of cell homogenate while lanes 7–11 were loaded with 1, 2, 4, 8, and 12 ng of purified L-cell ACBP. Lane 1, untransfected L-cells. Lane 2, untransfected LM cells (a cell line derived from L-cells). Lane 3, transfected L-cells expressing 0.4% of cytosolic protein as L-FABP. Lane 4, transfected L-cells expressing 0.35% of cytosolic protein as I-FABP. Lane 5, transfected L-cells expressing 0.036% of cytosolic protein as 13 kDa SCP-2. Lane 6, transfected L-cells expressing 0.03% of cytosolic protein as 15 kDa pro-SCP-2. Lanes 7–11, ACBP standards of 1, 2, 4, 8, and 12 ng, respectively.

0.04, 0.61 ± 0.01, and 0.73 ± 0.05 ng ACBP/10 μg homogenate protein, respectively. This is also consistent with studies on chronic ethanol administration, which results in fatty infiltration of the fatty liver. Chronic ethanol administration increases liver L-FABP expression (109) (Myers-Payne, S.C., Hubbell, T., Wood, W.G., and Schroeder, F., manuscript in preparation), without affecting liver ACBP expression (Myers-Payne, S.C., Hubbell, T., Wood, W.G., and Schroeder, F., manuscript in preparation) in the mouse. These studies suggest that cellular ACBP levels are not regulated by expression of L-FABP or I-FABP.

SCP-2. Although an early report indicated that total immunoreactive liver SCP-2 was relatively unresponsive to cholesterol feeding or lipid-lowering agents (83), more recent data show mRNA and SCP-2 levels vary over a 10- to 30-fold range under different development, hormonal, or lipid-lowering agent treatments (84,110–117). Expression is regulated at three levels: transcription of the gene (84,110–117), post-translational modification (118,119), and possibly alternative mRNA splicing (90,118,120). SCP-2 gene expression is developmentally regulated (113,117) and is modulated translationally and/or post-translationally by lipid-lowering agents (113,115). In addition, expression of the various molecular weight forms of SCP-2 appears differentially responsive depending on organs and subcellular organelle type. For example, in steroidogenic cells trophic hormones and steroids stimulated differential expression of mRNA transcripts and increased mitochondrial SCP-2 3-fold (110,112).

The observation that SCP-2 stimulates the formation of cholesteryl esters (84,121–125) and binds LCFA-CoA (Frolov, A., Cho, T.-Y., Billheimer, J.T., and Schroeder, F., submitted for publication) suggests that cellular SCP-2 levels could modulate the expression of ACBP. The effect of 13 kDa SCP-2 and 15 kDa pro-SCP-2 expression on expression of ACBP was examined in transfected L-cell fibroblasts. In L-cells transfected with cDNA encoding either 13 kDa SCP-2 or 15 kDa pro-SCP-2, only the 13 kDa SCP-2 protein is detectable in Western blots (126). This is due to the rapid post-translational cleavage of the amino terminal 20 amino acid leader sequence of the 15 kDa pro-SCP-2 protein. L-cells transfected with either 13 kDa SCP-2 or 15 kDa pro-SCP-2 differentially affect the uptake of cholesterol (126) and fatty acids (Murphy, E.J., Barhoumi, R., Frolov, A., Burghardt, R., and Schroeder, F., submitted for publication). However, neither 13 kDa SCP-2 nor 15 kDa pro-SCP-2 expression significantly altered ACBP expression in transfected L-cells (Fig. 2). ACBP levels in untransfected L-cells, LM-cells (derived from L-cells), transfected L-cells with 0.036% of cytosol protein as 13 kDa SCP-2, and transfected L-cells with 0.03% of cytosol protein as 15 kDa pro-SCP-2 were 0.83 ± 0.15, 0.72 ± 0.04, 0.64 ± 0.05, and 0.50 ± 0.05 ng/10 μg homogenate, respectively. This lack of significant difference is also consistent with studies on chronic ethanol administration where increases in mouse brain SCP-2 expression did not affect ACBP expression (82). These studies suggest that cellular ACBP levels are not regulated by expression of SCP-2.

STRUCTURE OF THE LCFA-CoA BINDING PROTEINS

Cytosolic FABP. The intracellular FABP are small, 14–16 kDa, cytosolic proteins. The tertiary structure of the FABP family has been extensively reviewed previously and will not be reiterated here (43). The common structural motif of these proteins consists of ten antiparallel beta strands oriented to form two sheets making up a slightly elliptical barrel. The beta strands of one sheet are perpendicular to those of the other sheet with the strands connected by two short helixes or by tight turns. This gives the FABP an overall three-dimensional clam shape. Although a gap exists between beta strands D and E, it apparently does not constitute the ligand entry portal. The interior of the beta barrel contains a hydrophobic ligand binding cavity. However, there are some charged amino acid residues and water molecules present in the binding cavity. X-ray crystallography indicates that fatty acid ligands are oriented with the fatty acid carboxyl bound deep within the binding pocket *via* a salt bridge to Arg residues and by hydrophobic interactions as well. Competition studies between long-chain fatty acids and LCFA-CoA indicate that these lig-

ands compete for the same binding sites in the liver FABP (41; Frolov, A., and Schroeder, F., submitted for publication). However, the orientation of LCFA-CoA bound to FABP is not known. Because of the size and polarity of the CoA portion of LCFA-CoA, this would preclude the entry of this portion of the molecule into the hydrophobic binding pocket (see section on ligand binding below). Based on Silicon Graphics (Mountain View, CA) computer modeling and the known structure of related FABP (127), a proposed structure of L-FABP and orientation of two LCFA-CoA in the binding sites have been developed (Fig. 1B). As shown, the CoA portion of the LCFA-CoA is too large and polar to be accomodated in the binding sites of L-FABP. Instead, the hydrophobic fatty acid alkyl chains of the LCFA-CoA are oriented with the methyl end of the fatty acyl groups buried deep in the L-FABP binding pockets.

ACBP. ACBP is a small protein, with 86 amino acids and a molecular weight of approximately 10 kd (19,68,92). The three-dimensional structure of ACBP from bovine liver and recombinant bovine ACBP expressed in *Escherichia coli* in solution (Fig. 3) has been elucidated using multi-dimensional nuclear magnetic resonance (NMR) spectroscopy (128). ACBP forms four alpha helixes, designated A1 through A4 (129–131). A2 forms the central point and has nonpolar contacts to the other three helices (131). A1 and A4 run antiparallel to A2 while A3 runs almost parallel to A2 (129–131). Hydrophobic interactions (hydrogen bonds) between the four alpha helices form a three-dimensional structure with the appearance of a "shallow bowl" or groove (130,131). The center outer and inner surfaces of the bowl are composed primarily of a cluster of hydrophobic amino acid residues while the rim of the bowl and upper outside surface contain a large number of charged amino acid residues from the N-terminal portions of A2 and A3 and the hydrophilic residues of A1 and A4 (130,131). The binding site for acyl-CoA esters involves the cluster of hydrophobic residues located in the bottom inner surface of ACBP of which the amino acid sequence from Asp^{21} to Asp^{38} appears to be important (132) (Fig. 3). This portion of the hydrophobic ligand binding domain in the second alpha helix (amino acid residues, 23–28) is highly conserved from yeasts to humans. The only charged amino acid residues in the ligand binding site are Lys 13 and Lys 32 on the inside surface and His 30 on the outside bottom (130). The binding site is arranged so that only LCFA-CoA esters can bind strongly (131) (Fig. 3). The fatty acyl chain, pantetheine, and Cys parts of the ligand fit into the binding site of ACBP by forming a combination of electrostatic and hydrogen bonds so that the nonpolar portions of the ligand are hidden and the polar portions are on the surface (131). The 3'phosphate group of the CoA portion of the ligand is essential for binding to ACBP (133). It forms salt bridges with the lysine residues and hydrogen bonds to tyrosine 28. Therefore, binding depends upon the nonpolar interactions between the ACBP binding site and the acyl chain, methyl groups, and pantetheine portions of the ligand as well as polar and nonpolar binding of the CoA to ACBP which protects the nonpolar portions of the ligand from solvent (131).



FIG. 3. Nuclear magnetic resonance (NMR) structure of recombinant bovine ACBP with bound LCFA-CoA. Silicon Graphics model based on previously published NMR data (129–131). The image was produced with a program developed by Jon A. Christopher in 1996, "SPOCK: the Structural Properties Calculation and Observation Kit" Program Manual, The Center for Macromolecular Design (Texas A&M University, College Station, TX). See Figure 2 for other abbreviation. See Figure 1 for company source.

SCP-2. The tertiary structure of the SCP-2 family is perhaps the least understood of the intracellular LCFA-CoA binding proteins. Only a single report on the NMR structure of the human 13.2 kDa SCP-2 holoprotein has appeared (139). Three alpha-helixes and a five-stranded beta-sheet, arranged in antiparallel fashion, constitute the polypeptide backbone of SCP-2. Deletion of either the amino-terminal amphipathic alpha-helix or deletion of the carboxyl terminal beta-sheet inactivates SCP-2 in terms of sterol and phospholipid transfer (90). Amino acid substitutions at Asn^{104} or near Cys^{71} also abolished SCP-2 sterol and phospholipid transfer activity (90). SCP-2 binds fatty acids (87). Forster energy transfer data indicate the SCP-2 fatty acid binding site, located on the opposite side of SCP-2 as the single tryptophan residue, may coincide with the SCP-2 site associated with sterol and phospholipid transfer (Frolov, A., Billheimer, J.T., and Schroeder, F., submitted for publication). Since LCFA-CoA compete for the same site as fatty acid binding to SCP-2 (Frolov, A., Cho, T.-Y., Billheimer, J.T., and Schroeder, F., submitted for publication), this would suggest that the LCFA-CoA may also coincide with the above activities. The effect of these mutations in SCP-2 on fatty acid or LCFA-CoA binding has not been examined. There are no reports on the tertiary structure of holo-SCP-2 with any bound ligand. Likewise the orientation of LCFA-CoAs bound to SCP-2 is not known. However, the size and polarity of the CoA portion of LCFA-CoA would preclude the entry of this portion of the

molecule into what appears to be a very hydrophobic fatty acid binding pocket in the 13.2 kDa SCP-2 (87). Nothing is known of the structural characteristics of the 58 kDa SCP-x.

LCFA-CoA BINDING CHARACTERISTICS OF MAMMALIAN LCFA-CoA

FABP. Although endogenous bound fatty acids have been reported in native FABP isolated from tissues and recombinant FABP isolated from bacteria, the presence of endogenous bound LCFA-CoA in these FABP preparations has not been documented (3,44). However, many early studies of native FABP from liver and heart demonstrated LCFA-CoA binding *in vitro* (1–5,44). L-FABP bound palmitoyl and oleoyl CoA with high affinity, K_d near 0.14–2.8 μM, depending on the specific LCFA-CoA and the assay used (Table 7). There is apparent disagreement on the stoichiometry of LCFA-CoA binding to L-FABP with the early studies with native L-FABP indicating 1:1 stoichiometry (1,2,41) while a later study with recombinant L-FABP showed stoichiometry of 2:1 (41). This apparent discrepancy was resolved by the finding that, due to the type of protein assay used, the early studies overestimated L-FABP by 1.7-fold (41,140,141). Native H-FABP isolated from human or rat heart also bound LCFA-CoA with high affinity, K_ds near 0.5 μM, but with less than molar stoichiometry (5). In contrast, the native adipocyte adipocyte lipid binding protein (ALBP) bound LCFA-CoA more weakly, with K_d near 10 μM, than either native L-FABP or native H-FABP (42) (Table 7).

The above observations using the Lipidex-1000 assay with native L-FABP, H-FABP, and ALBP isolated from liver, heart, and adipose tissues, respectively, to demonstrate LCFA-CoA binding were recently questioned on the basis of two issues (7). First, it was suggested that early preparations of native FABP were impure and cross-contaminated with ACBP such that copurified ACBP, but not FABP, accounted for LCFA-CoA binding ability of these FABP. Second, it was shown that LCFA-CoA binding to L-FABP (but not to ACBP) was highly dependent on the ratio of ligand/protein/Lipidex-1000 in the assay. In fact, with a modified Lipidex-1000 assay no binding of LCFA-CoA to L-FABP was demonstrated (7). This controversy was resolved through the use of recombinant L-FABP (41) and H-FABP (49). Since *E. coli* do not contain ACBP, there is no possibility of cross-contamination with this protein. The recombinant L-FABP bound both oleoyl CoA (Lipidex-1000 assay) and *cis*-parinaroyl CoA (fluorescence assay) with K_d in the range of 3–13 μM (41). The fluorescence assays are not a competition assay and therefore do not suffer from the problems intrinsic with a competition assay like the Lipidex-1000 assay. Furthermore, L-FABP has two fluorescent fatty acid binding sites (140,141) that are competitively displaced by oleoyl-CoA (41; Frolov, A., and Schroeder, F., submitted for publication; Jolly, C.A., Hubbell, T., Behnke, W.D., and Schroeder, F., submitted for publication). In addition, the K_d for binding of recombinant bovine L-FABP to oleoyl-CoA was determined to be 1 μM by titration microcalorimetry (142). The ability of recombinant H-FABP to bind LCFA-CoA was confirmed in a displacement assay where oleoyl CoA was able to displace bound fatty acid from recombinant H-FABP (49). These data clearly show that the recombinant L-FABP and H-FABP both bind LCFA-CoA, and suggestions that the native L-FABP and H-FABP do not bind LCFA-CoA are most likely due to the type of assay used.

Additional evidence indicates that several other members of the FABP family also bind LCFA-CoA (Table 7). The ability of recombinant brain FABP (B-FABP) to bind LCFA-CoA was confirmed in a displacement assay where oleoyl CoA was able to displace bound fatty acid from the recombinant B-FABP (49). Previously all reports on native and recombinant I-FABP ligand interaction concluded that I-FABP, unlike

TABLE 7
Fatty Acid Binding Proteins That Also Bind Fatty Acyl-CoA[a]

Protein	Source	Assay	Fatty acyl CoA	K_d (μM)	Stoichiometry (mole/mole)	Reference
			Fatty acid-binding protein family			
L-FABP	Rat liver	Lipidex	Oleoyl-CoA	1.2–2.0	1	1–3
L-FABP	Rat liver	Lipidex	Palmitoyl-CoA	0.14–2.8	—	2–4
L-FABP	r-Rat liver	Lipidex	Oleoyl-CoA	3.6 ± 0.6	2.4 ± 0.3	41
L-FABP	r-Rat liver	Fluorescence	*cis*-Parinaroyl-CoA	13.7 ± 1.7	1.8 0.5	41
L-FABP	r-Bovine liver	Titration microcalorimetry	Oleoyl-CoA	1.0 ± 0.1	1.3	142
H-FABP	Human heart	Lipidex	Palmitoyl-CoA	0.5 ± 0.1	0.24 ± 0.03	5
	Rat heart	Lipidex	Palmitoyl-CoA	0.5 ± 0.0	0.30 ± 0.06	5
B-FABP	r-Mouse heart	Lipidex	Oleoyl-CoA	Displacement assay		49
	r-Mouse brain	Lipidex	Oleoyl-CoA	Displacement assay		49
I-FABP	r-Rat intestine	Tryptophan fluorescence	Oleoyl-CoA	2.3 ± 0.5	1	*b*
ADLP	Rat adipocyte	Lipidex	Oleoyl-CoA	10	1	42
			Sterol carrier protein-2- family			
13 kDa SCP-2	r-Human liver	Fluorescence	*cis*-Parinaroyl-CoA	0.08	1	Figure 5
58 kDa SCP-x	r-Rat liver	Thiolase enzyme	3-Oxoacyl-CoA	—	—	172

[a]See Table 5 for abbreviations. [b]Jolly, C.A., Hubbell, T., Behnke, W.D., and Schroeder, F., submitted for publication.

the other members of the FABP family, had a strict ligand specificity for long-chain fatty acids. Consistent with this and in contrast to L-FABP, an initial report showed negligible increase in fluorescence intensity upon addition of *cis*-parinaroyl CoA to recombinant I-FABP (41). However, more recent data using intrinsic I-FABP, tryptophan, and acrylodated-I-FABP fluorescence assays showed that oleoyl-CoA bound to I-FABP with high affinity, K_d near 0.2–2.3 μM, depending on the assay used (Jolly, C.A., Hubbell, T., Behnke, W.D., and Schroeder, F., submitted for publication). The reason for the discrepancy with the earlier study is that I-FABP appears to have a unique LCFA-CoA binding site in which the *cis*-parinaroyl group of *cis*-parinaroyl CoA does not enter deeply into the hydrophobic portion of the ligand binding pocket (Jolly, C.A., Hubbell, T., Behnke, W.D., and Schroeder, F., submitted for publication). Additional supportive evidence for LCFA-CoA binding to I-FABP was obtained by phase and modulation resolved fluorometry. Oleoyl-CoA significantly decreases the lifetime of I-FABP tryptophan by 17% ($P < 0.05$), decreases the limiting anisotropy of I-FABP tryptophan by 28% ($P < 0.02$), but does not change the hydrodynamic radius of I-FABP (Frolov, A., and Schroeder, F., submitted for publication).

In summary, at least five members of the FABP family (L-FABP, H-FABP, ALBP, B-FABP, and I-FABP) bind LCFA-CoA with μM dissociation constants and (with the exception of L-FABP which binds two LCFA-CoA per molecule of protein) with binding stoichiometries of 1:1 (Table 7). As mentioned above, these high affinities as well as the near millimolar cytosolic concentration of FABP (ranging from 1–6% of cytosolic protein) makes the FABP important potential contributors to LCFA-CoA binding in cytosol of tissues such as liver, heart, adipose, and intestine.

ACBP. ACBP isolated with an endogenous bound LCFA-CoA has not been reported. However, numerous studies *in vitro* demonstrate LCFA-CoA binding to a single binding site on ACBP (Table 8). NMR studies indicate that apo-ACBP and holo-ACBP (containing bound palmitoyl CoA) are structurally almost identical (128). Therefore, ligand binding does not induce a significant change in the tertiary structure of ACBP. However, the presence of ligand in the binding site results in a decrease in flexibility of the ACBP protein backbone within the binding site (128). Additional evidence indicates that ACBP specifically binds acyl CoA esters and not free fatty acids, free CoA, ATP, NADH, NADPH, GABA, palmitoyl carnitine, or cholesterol (8,19,68,143). The 3′ phosphate group on the ribose moiety of the acyl CoA ester is essential for binding since 3′-dephospho-hexadecanoyl-CoA does not displace 12-doxylstearoyl-CoA (143). Also, 3′-dephospho-doxylstearoyl-CoA does not bind recombinant bovine ACBP when analyzed by electromagnetic spin resonance spectroscopy (143). Therefore, both the CoA portion of the ligand as well as the acyl chain are involved in binding of LCFA-CoA to ACBP.

ACBP binds acyl-CoA esters with an acyl chain length of 8–22 carbons with high affinity, but binding affinity has been reported to be greatest for long-chain acyl-CoA esters with acyl chains of 14–22 carbons (19,143). The affinities of ACBP for a variety of LCFA-CoA is highly dependent on the type of assay used to determine binding. With the Lipidex 1000 binding assay, it was reported that bovine and rat ACBP bound acyl-CoA esters with a K_d near 0.13–0.27 μM (Table 8). [It should be noted that under the Lipidex-1000 assay conditions used therein, binding of LCFA-CoA to liver or heart FABP was not observed (8).] ACBP binds to acyl-CoA esters with equimolar stoichiometry (8,19). The fluorescent *cis*-parinaroyl CoA assay yielded a K_d of 0.2 μM (Table 8). The latter assay is not a competition assay and not subject to the same potential artifacts as the Lipidex-1000 assay. In contrast to the μM K_d reported with the Lipidex 1000 and fluorescence assays, other assays suggest that ACBP binds LCFA-CoA with 10^2- to 10^7-fold lower K_d. One study reported that the binding affinity of ACBP to long-chain acyl CoA esters is so high that no free ligand is detected, and therefore no dissociation constant can be determined by electromagnetic spin resonance spectroscopy (143). Another report using titration microcalorimetry showed K_d values for binding of hexadecanoyl CoA to

TABLE 8
Acyl CoA-Binding Protein (ACBP) Ligand Affinities

Type	Ligand	Technique	K_d (M)	B_{max}	Reference
Rat liver	Palmitoyl-CoA	Lipidex	0.27×10^{-6}	0.95	8
Bovine liver	Palmitoyl-CoA	Lipidex	0.22×10^{-6}	1.02	8
Rat liver	Oleoyl-CoA	Lipidex	0.14×10^{-6}	0.95	8
Bovine liver	Oleoyl-CoA	Lipidex	0.12×10^{-6}	0.87	8
Rat liver	*cis*-Parinaroyl-CoA	Fluorescence	0.20×10^{-6}	1.0	41[a]
r-Bovine liver	Octanoyl-CoA	Titration calorimetry	0.24×10^{-6}		144
	Lauryl-CoA	Titration calorimetry	0.65×10^{-8}		144
	Palmitoyl-CoA	Titration calorimetry	0.45×10^{-13}		144
r-Bovine liver	12-Doxylstearoyl-CoA	ESR	—	1.0	143
L-cell fibroblast	*cis*-Parinaroyl-CoA	Fluorescence	0.4×10^{-6}	1.0	*b*
L-cell fibroblast	Oleoyl-CoA	Lipidex	0.3×10^{-6}	1.3	*b*

[a]Reference 41; Jolly, C.A., Hubbell, T., Behnke, W.D., and Schroeder, F., submitted for publication; ESR, electron spin resonance. [b]Gossett and Schroeder (manuscript in preparation).

recombinant bovine ACBP to be in the range 0.45–0.2 × 10^{-13} M (144). In addition, two yeast ACBP homologues which bind LCFA-CoA with high affinity have been characterized and the K_d determined by titration calorimetry was 0.55 × 10^{-10} (145). These picomolar K_d obtained by microcalorimetry differ by 2–7 orders of magnitude from those obtained with the Lipidex-1000 (0.1–0.2 μM) or *cis*-parinaroyl CoA (0.2 μM) assays (Table 8). Although the Lipidex-1000 assay does not give accurate binding constants for ACBP since it can extract acyl-CoA esters bound to Lipidex, it is difficult to imagine that ACBP could serve as a LCFA-CoA donor *in vivo* if such high affinities obtained by titration microcalorimetry actually reflect the *in vivo* situation. Further work will be necessary to resolve the differences obtained with the various methods.

SCP-2. Of all the intracellular lipid binding proteins, perhaps the most difficult one for demonstrating ligand binding has been the SCP-2. Endogenous bound LCFA-CoA has not been reported. Even *in vitro* studies are few and subject to problems. This issue has been at least partly resolved by the recent discovery that SCP-2 ligand interactions are very sensitive to the solvent vehicle used to add the ligand *in vitro* (87). The latter work clearly demonstrated that, in the absence of solvent 12-*N*-methyl-(7-nitrobenz-2-oxa-1,3-diazol)amino stearate (NBD-stearic acid) bound to SCP-2 with high affinity, K_d near 0.2 μM. NBD-stearic acid is poorly fluorescent in aqueous buffer. However, in the presence of SCP-2, NBD-stearic acid fluoresces strongly (Fig. 4, top curve). As indicated by decreased fluorescence of bound NBD-stearic acid, addition of oleoyl-CoA results in displacement of bound NBD-stearic acid (Fig. 4, bottom curve). More direct evidence for LCFA-CoA binding to SCP-2 was obtained with *cis*-parinaroyl CoA. *Cis*-parinaroyl CoA is poorly fluorescent in aqueous buffer. However, when bound to SCP-2, it fluoresces strongly and a saturation binding curve may be constructed (Fig. 5). Analysis of the binding curve for SCP-2 binding to *cis*-parinaroyl CoA yields a K_d of 0.08 μM (Table 7).

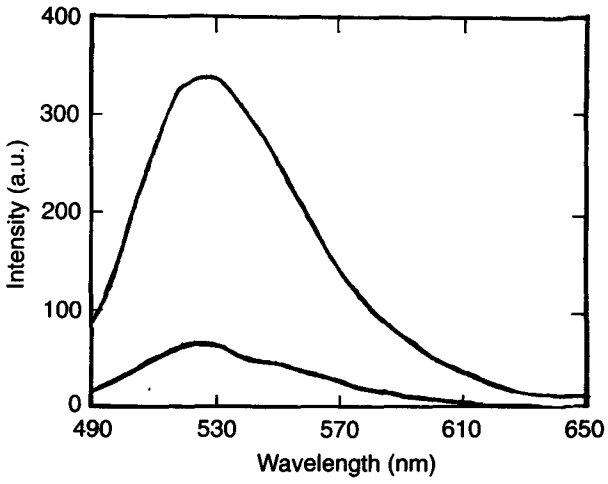


FIG. 4. Displacement of sterol carrier protein-2 (SCP-2) bound NBD-stearic acid by oleoyl CoA. All spectroscopic measurements were as described elsewhere (Frolov, A., Cho, T.-Y., Billheimer, J.T., and Schroeder, F., submitted for publication). NBD-stearic acid (0.1 μM) was added to SCP-2 (0.1 μM) (top curve). Thereafter, oleoyl-CoA (5 μM) was added (bottom curve). In both curves a blank, low fluorescence intensity of NBD-stearic acid in pH 7.4 phosphate buffer was subtracted. Excitation wavelength was 466 nm. Fluorescence intensity was determined at 22°C.

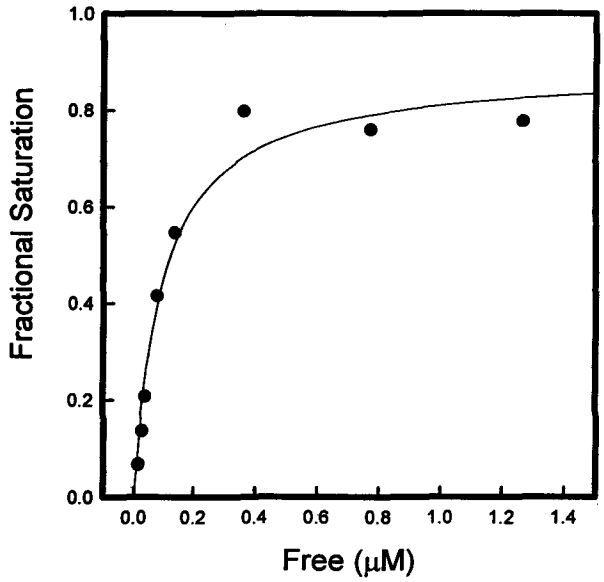


FIG. 5. *Cis*-parinaroyl CoA binding to SCP-2. All spectoscopic measurements were as described elsewhere (Frolov, A., Cho, T.-Y., Billheimer, J.T., and Schroeder, F., submitted for publication). Increasing SCP-2 was added to 0.3 μM *cis*-parinaroyl CoA in phosphate buffered pH 7.4 saline. Excitation was at 313 nm and fluorescence emission intensity was measured at 420. See Figure 4 for abbreviation.

Relative distribution of LCFA-CoA among the cytosolic FABP, ACBP, and SCP-2. At least three types of nonenzymatic cytosolic proteins interact with LCFA-CoA: FABP, ACBP, and SCP-2. Using the *cis*-parinaroyl CoA fluorescence binding assay, a rank order of K_d may be constructed as follows: FABP, 13 μM; ACBP, 0.2 μM; and SCP-2, 0.08 μM (Tables 7 and 8). LCFA-CoA binding to SCP-2 has not been determined by the Lipidex-1000 assay or titration microcalorimetry. However, both of these assays indicate that the rank order of K_d is still maintained for FABP and ACBP (Lipidex-1000: FABP = 0.14–3.6 μM and ACBP = 0.12–0.27 μM; Titration microcalorimetry: FABP = 1 μM and ACBP = 0.24 × 10^{-6} to 0.45 × 10^{-13} M) (Tables 7 and 8).

As pointed out in the preceeding sections, four factors will influence the amount of LCFA-CoA bound to the three types of LCFA-CoA binding proteins in the cell: (i) the concentration and subcellular distribution of LCFA-CoA; (ii) the concentration and subcellular distribution of LCFA-CoA binding proteins; (iii) the LCFA-CoA binding affinities of the three LCFA-CoA binding protein families; and (iv) the LCFA-CoA chain length specificity of each binding protein. This creates difficulty in accurately predicting the proportion of LCFA-

CoA bound to the individual members. For example, the preceding sections pointed out that the majority of LCFA-CoA is membrane-bound and the remaining LCFA-CoA is almost all bound to cytosolic proteins. Among these proteins, the FABP have the highest concentration in the cell cytosol [5- to 15-fold higher than ACBP in liver; 2- to 51-fold higher than ACBP in heart (19,146)]. Although "cytosolic" ACBP and SCP-2 concentrations appear in the same range, the preceding sections pointed out that SCP-2 is largely membrane-associated and may be very concentrated in peroxisomes where SCP-x appears located in the peroxisomal matrix while the SCP-2 may be localized on the cytofacial leaflet of the peroxisome. In addition, there is a lack of agreement between microcalorimetric and other methods reporting K_d for LCFA-CoA binding to the three proteins. Finally, the LCFA-CoA chain length specificity is not documented for all of these binding proteins. ACBP has highest affinities for long-chain LCFA-CoA. The chain length preferences of SCP-2 and L-FABP for long-chain fatty acids would also suggest that the LCFA-CoA would bind better than the short- or medium-chain fatty acyl-CoA. Nevertheless, one may estimate the range of relative distribution of protein-bound LCFA-CoA among the three species (FABP, ACBP, and SCP-2) in the cell cytosol. If the higher cellular content of FABP over ACBP and SCP-2 is considered to be offset by the higher affinity of the latter two proteins for LCFA-CoA, then all three protein fractions may contain roughly similar quantities (but at different occupancies of available binding sites) of LCFA-CoA. At least one study suggests that both the FABP and ACBP play a role in acyl-CoA binding *in vivo* in liver and heart (8). If the microcalorimetrically determined affinites (seven orders of magnitude higher affinities for LCFA-CoA) are assumed correct, then essentially all of the cytosolic LCFA-CoA would be bound by ACBP. Indeed, under some assay conditions *in vitro,* it has been reported that ACBP can bind all acyl CoA in the presence of FABP (8).

FUNCTION OF CYTOSOLIC LCFA-CoA BINDING PROTEINS

Cytosolic FABP. There is no evidence obtained *in vivo* for the physiological role of FABP binding LCFA-CoA. However, a number of studies *in vitro* show that FABP alters the activities of a variety of lipid synthetic and oxidative enzymes that utilize LCFA-CoA as substrates (Table 9). For example, L-FABP enhances or inhibits microsomal fatty acyl-CoA synthase (1,44,147) and stimulates phosphatidic acid biosynthesis *in vitro* (1,147–150). Although early data with unwashed microsomes suggested that I-FABP did not stimulate phosphatidic acid synthesis (41), more recent data from our laboratory using washed microsomes indicate that I-FABP does stimulate phosphatidic acid biosynthesis (Jolly, C.A., Hubbell, T., Behnke, W.D., and Schroeder, F., submitted for publication). Apparently the unwashed liver microsomes contain enough LCFA-CoA binding proteins to raise the baseline enzymatic activity and obscure effects of I-FABP. As pointed out above, the suggestion that the lipid synthetic stimulatory activities in early preparations of native FABP may have been due to copurified ACBP is unlikely. This issue was clearly resolved in several recent studies demonstrating that recombinant L-FABP stimulates incorporation of LCFA-CoA into phosphatidic acid *in vitro* (41), into phospholipids in intact transfected cells expressing L-FABP (108), into cholesteryl esters *in vitro* (151), and into cholesteryl esters in intact transfected cells expressing L-FABP (151). Likewise, recombinant I-FABP stimulated incorporation of fatty acids into triglycerides and cholesteryl esters in intact transfected cells expressing I-FABP (152,153).

In addition to stimulating enzymes using LCFA-CoA as substrates, the FABP alter fatty acid synthesis and other proteins whose activities are regulated by LCFA-CoA (Table 10). In only one case was inhibition observed, e.g., fatty acid synthetase is inhibited by FABP (6). In contrast, several lipogenic

TABLE 9
Effect of Long-Chain Fatty Acyl-CoA (LCFA-CoA) Binding Proteins on Activities of Lipid Oxidative and Synthetic Enzymes Using LCFA-CoA as a Substrate[a]

Process	Enzyme	FABP	ACBP	SCP-2	Reference
LCFA-CoA synthesis	LCFA-CoA synthetase				
	Mitochondrial	L-FABP (+)	+	NA	12,144
	Microsomal	L-FABP, I-FABP (+/–), Lu-FABP (+)	+	NA	12,44[b],144,217 216
LCFA-CoA β-oxidation	Carnitine palmitoyl-transferase	L-FABP (+)	+	NA	144,157
	LCFA-CoA hydrolase	L-FABP (+)	+	NA	6,12,147
Phospholipid synthesis	Glycerophosphate acyltransferase	L-FABP, H-FABP, I-FABP (+)	+	NA	41,144,147–150,218,219[b,c]
	Monoacylglycerolphosphate acyltransferase	L-FABP, H-FABP (+)	NA	NA	41,144,218,219
	Acyl-CoA/lysophospholipid acyltransferase	L-FABP, I-FABP (+)	+	+	72[c]
Cholesterol esterification	Acyl-CoA/cholesterol acyltransferase	L-FABP (+)	NA	+	122,125,221–224

[a]NA = not assessed; Lu-FABP = lung FABP. See Table 5 for other abbreviations. [b]Jolly, C.A., Hubbell, T., Behnke, W.D., and Schroeder, F., submitted for publication. [c]Jolly and Schroeder (manuscript in preparation); +, increase; +/–, increase/decrease.

TABLE 10
Effect of LCFA-CoA Binding Proteins on the Activities of Proteins Regulated by LCFA-CoA[a]

Process	Protein	FABP	ACBP	SCP-2	Reference
Fatty acid synthesis	Fatty acid synthetase	L-FABP (–)	+	NA	6
	Acetyl-CoA carboxylase	L-FABP (+)	+	NA	12,154
	Glucose-6-phosphate dehydrogenase	P-FABP, L-FABP, Lu-FABP (+)	NA	NA	225–227
	Malate dehydrogenase	P-FABP (+)	NA	NA	220
Transport	Adenine nucleotide transporter	L-FABP (+)	+	NA	12,154
	Na^+ dependent amino acid transporter[b]	L-FABP (+)	NA	NA	228

[a]NA = not assessed; P-FABP = placental FABP. See Tables 5 and 9 for abbreviations. [b]Regulated by LCFA; +, increase; –, increase/decrease.

enzyme activities were stimulated by L-FABP, Lu-FABP, or P-FABP (Table 10). For example, free LCFA-CoA esters are potent inhibitors of specific enzymes within the cell such as acetyl-CoA carboxylase (ACC) and adenine nucleotide translocase (ANT) which are of vital importance for cellular function. LCFA-CoA esters inhibit ANT and therefore suppresses mitochondrial respiration and ATP synthesis while inhibition of ACC suppresses fatty acid synthesis. *In vitro* examination of the effects of L-FABP upon LCFA-CoA inhibition of ACC showed that in the presence of LCFA-CoA the ACC and ANT activities were stimulated by L-FABP (12,154).

ACBP. The physiological function of ACBP is not known. However, a number of studies both *in vitro* and *in vivo* show that ACBP is involved in lipid metabolism and in the regulation of long-chain acyl CoA levels within the cell (Table 9). In support of this potential role of ACBP in basal cellular metabolism, the gene encoding this protein has features of a housekeeping gene such as: the promoter region located in a CpG island, lack of a TATA box, and the presence of processed pseudogenes (65). Additional support of the role of ACBP as a housekeeping protein is its stability in several cell lines *in vitro*. The half-life of the ACBP protein within 3T3-L1 and 3T3-C1 mouse embryo fibroblasts, H-4-11-E rat hepatoma cells, and HepG2 human hepatoma cells *in vitro* are approximately 25–53 h (155). Recent evidence has proven that a major function of ACBP in yeast is to maintain a pool of bound intracellular LCFA-CoA. Recombinant bovine ACBP transfected into yeast *(Saccharomyces cervisiae)* increased the total ACBP and resulted in an increase in the pool of long-chain acyl CoA esters, possibly *via* the mechanism of binding LCFA-CoA and decreasing feedback inhibition of yeast fatty acid synthetase and LCFA-CoA synthetase (156). In addition, overexpression of yeast ACBP *(S. carlsbergensis)* resulted in subsequent expansion of the intracellular acyl-CoA pool (145).

ACBP from bovine liver was first discovered to induce the synthesis of medium-chain fatty acyl-CoA esters by goat mammary gland fatty acid synthetase *in vitro* by binding to acyl CoA esters (6). Research involving the *in vitro* effects of ACBP upon other enzymes involved in lipid metabolism within the cell has shown that ACBP stimulates the synthesis of LCFA-CoA esters by mitochondrial LCFA-CoA synthetase (12). Newly synthesized LCFA-CoA were removed from the mitochondrial membrane by ACBP and therefore reduced the negative feedback of LCFA-CoA esters upon the enzyme (12). However, saturation of ACBP with long-chain acyl CoA esters abolished the stimulatory effect of ACBP upon acyl CoA synthetase (12). In addition, ACBP increases the activity of outer mitochondrial carnitine palmitoyl transferase, the enzyme which is involved in fatty acid oxidation in the mitochondria (157). The enzyme, carnitine palmitoyltransferase, is located on the outer mitochondrial membrane facing the cytosol and is particularly sensitive to the effects of micromolar concentrations of free LCFA-CoA esters such as palmitoyl-CoA. Bhuiyan and Pande (157) showed that various carnitine palmitoyl transferase preparations *in vitro* are able to use palmitoyl-CoA bound to ACBP in a near physiologic molar ratio of 1:1. Results of these experiments suggest that ACBP binds LCFA-CoA esters to prevent their inhibition of carnitine palmitoyl transferase, and at the same time ACBP facilitates removal of the acyl carnitine product from the enzyme so as to decrease feedback inhibition by the product. The latter suggestion is puzzling in view of other data (see above), indicating that ACBP does not bind palmitoyl carnitine. In addition, ACBP may sequester LCFA-CoA esters and facilitate their transport to the catalytic site of selected enzymes. Studies which support the potential role of ACBP in acyl-CoA transport have shown that ACBP can extract LCFA-CoA from phosphatidylcholine membranes immobilized on nitrocellulose and, subsequently, the ACBP–LCFA-CoA complex was able to transport and donate the LCFA-CoA to mitochondria or microsomes in suspension for fatty acid β-oxidation or glycerolipid synthesis (144).

Both cytoplasmic and microsomal membranes contain high levels of acyl-CoA hydrolases. Upon addition of LCFA-CoA to microsomal membranes followed by an incubation period at 37°C, only 10% of LCFA-CoA could be recovered in the absence of ACBP. In the presence of ACBP, the recovery of LCFA-CoA under the same conditions was 86% of added LCFA-CoA (12). Clearly ACBP protected LCFA-CoA from hydrolysis under these conditions. In contrast, L-FABP was not able to prevent LCFA-CoA hydrolysis under the same conditions (12). This would suggest that ACBP, but not L-FABP, may protect long-chain acyl-CoA esters from hydrolysis *in vitro*. It is important that this observation may not necessarily extend to other types of FABP.

ACBP is present in human red blood cell cytosol but not in the erythrocyte membrane (72). It appears to form a cytosolic reserve pool of LCFA-CoA for acylation of membrane phospholipids by lysophospholipid acyltransferase. ACBP-bound LCFA-CoA is preferred over free LCFA-CoA as a sub-

strate and therefore is important in the cellular distribution and utilization of LCFA-CoA as well as in phospholipid turnover in red blood cells (72).

As pointed out above, free LCFA-CoA esters are potent inhibitors of specific enzymes within the cell such as ACC and ANT which are of vital importance for cellular function. LCFA-CoA esters inhibit ANT and therefore suppress mitochondrial respiration and ATP synthesis while inhibition of ACC suppresses fatty acid synthesis. *In vitro* examination of the effects of ACBP upon LCFA-CoA inhibition of ACC showed that ACC and ANT activity were unaffected by LCFA-CoA esters as long as the molar ratio of LCFA-CoA ester to ACBP was less than 0.8 (12). In addition, ACBP has a higher affinity for LCFA-CoA esters because addition of ACBP to ACC bound to LCFA-CoA restored the activity of ACC (12). Due to the high binding affinity of ACBP for acyl-CoA esters and the high hydrolytic activity in cytosol and membranes, ACBP maintains a pool of acyl-CoA, and the intracellular free LCFA-CoA concentration *in vivo* is very low so as to maintain the activity of important enzymes such as ACC and ANT.

Free long-chain acyl-CoA esters readily partition into the phospholipid bilayer of membranes or bind membrane proteins and may therefore contribute to membrane damage by a detergent effect. ACBP, not FABP, prevented the binding of long-chain acyl-CoA ester to heat denatured microsomal membranes *in vitro* (8). These results suggest that ACBP may play a role in protecting membranes against damage by long-chain acyl CoA esters.

In addition to its role in relationship to acyl-CoA metabolism (see above), ACBP has been shown to effect a variety of other processes within cells. There has been considerable controversy regarding the function of ACBP in the brain. Two distinct types of benzodiazepine receptors have been identified in the brain. Central benzodiazepine receptors are present primarily in neurons and associated with GABA adrenergic ($GABA_A$) receptors which are ligand-gated chloride channels (158). Benzodiazepines such as diazepam and clonazepam bind these receptors with high affinity (158,159). ACBP has been shown to modulate this receptor in a fashion which can be blocked by antagonists and therefore was first thought to be a neurotransmitter (7,92,158). However, this question has not been fully resolved because actual direct binding of ACBP to the $GABA_A$ receptor has not been proven, purified ACBP from bovine liver does not have DBI activity in the brain, ACBP is present in very low levels in neurons, and micromolar concentrations of ACBP are required for inhibition of benzodiazepine binding when most neutrotransmitters are active at nanomolar concentrations (67,78,92,159,160).

The second type of benzodiazepine receptor, peripheral benzodiazepine receptors (PBR), are found in the brain and most peripheral tissues and are localized primarily in the outer mitochondrial membranes in many cells although other locations have been identified (161–164). These receptors mediate some of the pharmacologic effects of benzodiazepines, and are often abundant in steroid-producing tissues such as brain, adrenal cortex, and testis. Extensive work has identified the importance of these receptors in steroid biosynthesis.

ACBP undergoes posttranslational proteolytic cleavage resulting in biologically active cleavage products having varying effects in the brain and other tissue types (50,73,74, 165–167). Two peptide cleavage products of ACBP are octadecaneuropeptide (ODN), composed of amino acids 33–50 and triacontatetraneuropeptide (TTN), consisting of amino acids 17–50 (166). ODN appears to be involved primarily in modulation of GABA receptors in various tissue, and it also inhibits glucose-induced insulin secretion in the pancreas with a potency similar to intact ACBP (73,168). ACBP and its proteolytic cleavage products have been identified in tissues involved in steroid biosynthesis and have been shown to modulate steroid synthesis in the brain, adrenal gland, and testis (134–137,169,170) and regulate glucose-induced insulin secretion (73,133,168). It is still unclear whether intact ACBP, its processing products, or both are involved in modulation of PBR. Work in our laboratory indicates that an 8 kDa fragment of ACBP lacking the carboxy terminus did not affect the binding of ligand to the PBR (Gossett, R.E., and Schroeder, F., manuscript in preparation). However, ACBP and/or other proteolytic processing products of ACBP may be important in modulation of PBR in mitochondrial steroidogenesis. One study demonstrated that binding (chemical crosslinking) of purified, radiolabeled ACBP to mitochondrial PBR protein occurred in rat Leydig R2C cells and promoted constitutive steroidogenesis (170). In addition, ACBP depletion by treatment of the cells with cholesterol linked deoxynucleotide antisense to ACBP suppressed the expression of ACBP to undetectable levels by immunoblotting and resulted in a dramatic decrease in steroid synthesis (171). Both ODN and ACBP were detected in these cells, but which protein(s) bound PBR was not determined. Further work will be necessary to more fully characterize the role of ACBP and its cleavage products in modulation of PBR in various tissues.

SCP-2. The function of SCP-2 in LCFA-CoA metabolism or regulation is not well known at this time (Tables 9 and 10). However, there is strong indication that SCP-2 stimulates microsomal acyl CoA/cholesterol acyltransferase *in vitro* (Table 9). Whether it does so by enhancing incorporation of sterol or of LCFA-CoA or both remains to be resolved. There are some suggestions that SCP-2 may also be involved in peroxisomal β-oxidation of fatty acids. As pointed out in preceding sections, the 13.2 kDa form of SCP-2 binds LCFA-CoA and is found in high content in peroxisomes as well as other organelles. The 58 kDa SCP-x is found exclusively in peroxisomes (88). Furthermore, the 58 kDa SCP-x is a thiolase that binds and hydrolyzes long-chain 3-oxoacyl CoA, an intermediate in fatty acid β-oxidation (172).

PATHOBIOLOGY OF LCFA-CoA BINDING PROTEINS

FABP. The effect of disease on expression of FABP has been reviewed earlier and will not be covered in depth here (44).

Briefly, FABP expression is generally decreased in starvation, diabetes, and hepatitis depending on tissue type and type of FABP being examined. In contrast, FABP levels are increased in ischemia and chronic alcoholism. Interestingly, FABP is markedly reduced in several liver (44) and intestinal tumors (173) as well as transformed cell lines in culture: hepatomas (44,174,175), L-cells (151), Caco-2 (89,176,177). Furthermore, at least one FABP isolated from mammary gland and milk is closely related to H-FABP and has been identified as a potent mammary tumor inhibitor (178,179). Finally, liver L-FABP has been postulated to be involved in gene regulation and carcinogenesis (48,174,175,180,181).

ACBP. ACBP has been shown to be increased in a number of different disease states affecting the central nervous system. Increased ACBP concentrations have been detected in the cerebrospinal fluid of humans with chronic hepatic encephalopathy, and in patients with depression and schizophrenic behavior (182–184). In addition, there may be an association of ACBP levels with the development of alcohol dependence and withdrawal symptoms. Injections of ACBP into the lateral ventricles of rats elicited behavioral changes similar to those of alcohol withdrawal, and the expression of ACBP mRNA increased in mice inhaling ethanol vapor for eight days as well as up to three days after withdrawal of ethanol (51,185). Perhaps ACBP and/or a proteolytic peptide derived therefrom may by acting as a benzodiazepine receptor antagonist in these studies. Nicotine exposure for 24 h resulted in a dose-dependent increase in ACBP expression in mouse primary cultured cerebral cortical neurons (186). This effect lasted for approximately 48 h and was abolished by a nicotine acetylcholine receptor antagonist.

Increased ACBP expression has been demonstrated in a number of different tumor types *in vivo* such as astrocytomas, glioblastomas, and medulloblastomas with the highest expression occurring in the most anaplastic tumors (187,188). ACBP levels were low to undetectable in normal brain and benign brain neoplasms such as meningiomas and pituitary adenomas (187,188). In addition, PBR are low to undetectable in normal brain and low-grade astrocytomas whereas malignant tumors expressed high levels. In both astrocytomas and gliomas as well as cultured primary astrocytes and C-6 glioma cells, many cells expressing ACBP also expressed PBR (187,188). Other tumor cells in which ACBP has been identified include L cells (Fig. 2), ras or sre transformed immortalized rat colon cell lines (Gossett, R., Schroeder, F., Roths, J., Gunn, J.M., and Kier, A., manuscript in preparation), human prostatic adenocarcinoma cell lines, Leydig cell-derived tumor cell lines, rat insulinoma cell line, dimethylbenz(x)anthracene-induced mammary tumors in the rat, and in the supernatant from a metastatic human malignant melanoma cell line (77,138,170,189,190). The mechanistic basis for this association of ACBP expression with malignancy is, however, unclear at this time. Further work will be necessary to determine if ACBP is involved in the regulation of cell growth possibly *via* interaction with PBR. Alternately increased ACBP levels may be secondary to increased acyl CoA turnover in more rapidly growing cells.

SCP-2. SCP-2 expression is dramatically modulated in several disease states. Diabetic rat liver has a 4.5-fold higher 58 kDa SCP-2 (peroxisomal) and 60–90% lower 13.2 SCP-2 (cytosolic/peroxisomal) (114,116). In contrast, diabetes increased intestinal 13.2 kDa SCP-2 3- to 4-fold (116). Transformed cells had 10-fold lower SCP-2 levels than corresponding normal tissue (125,151,191). In addition, adrenal carcinomas exhibit decreased expression of SCP-2 mRNA compared to normal adrenal tissue (192). SCP-2 levels are also markedly reduced in peroxisomal deficiency (reviewed in Refs. 193–196) and other diseases (reviewed in Refs. 110,197).

CONCLUSIONS AND FUTURE DIRECTIONS

The ligand specificity and function of the LCFA-CoA binding proteins (FABP, ACBP, SCP-2) represent a controversial area of investigation. Much of the difficulty lies in the type of ligand binding assays used as well as the differential susceptibility of these proteins to the effects of assay conditions, i.e., different Lipidex-1000 assay conditions, the presence of solvents, etc. Further contributing to the confusion is the potential for mutual cross-contamination of these small proteins whose molecular weights are in the range of 10–15 kDa. Finally, the relationship of ACBP's ability to bind LCFA-CoA and influence LCFA-CoA metabolism to its putative interactions with benzodiazepine receptors and GABA receptors remain to be clarified.

What has been clearly established, however, is that mammalian cytosol contains at least three distinct families of LCFA-CoA binding proteins (FABP, ACBP, and SCP-2) and that members of all three families bind LCFA-CoA. Equally important, potential functions of this LCFA-CoA binding by individual members of these families have begun to be investigated. Because of the potential for many early preparations of FABP being cross-contaminated with ACBP, early reports of FABP functions in LCFA-CoA metabolism, and regulation of protein function need to be viewed with caution and should be re-evaluated. The use of recombinant L-FABP, however, has clearly shown that this protein binds LCFA-CoA and stimulates microsomal glycerophosphate acyltransferase and acyl CoA/cholesterol acyltransferase. Because of the newness of the discovery that SCP-2 binds LCFA-CoA, the role(s) of SCP-2 in LCFA-CoA metabolism and function are largely unknown. However, both native and recombinant SCP-2 stimulate microsomal acyl CoA/cholesterol acyltransferase.

While much work needs to be done delineating the functions of FABP and SCP-2 in LCFA-CoA metabolism, considerably more progress has been made in our understanding of ACBP function in LCFA-CoA metabolism. ACBP has been shown to specifically bind only LCFA-CoA esters and function as an LCFA-CoA pool former (6–8,19,68,92,143,145). Therefore, ACBP appears to play a key role in LCFA-CoA sequestration, transport, and utilization within cells by regulating the size of the intracellular LCFA-CoA pool, acyl CoA ester transport to and from enzymes involved in lipid metabolism, protection of enzymes whose activity is inhibited by

free LCFA-CoA esters, prevention of the breakdown of LCFA-CoA esters by intracellular hydrolases, and protection of cellular membranes.

Although no evidence exists at this time, by virtue of binding LCFA-CoA the FABP, ACBP, and SCP-2 may also play a regulatory role in the cell. LCFA-CoA esters are important for reactions involving protein acylation/deacylation while specific LCFA-CoA esters (myristoyl-CoA and palmitoyl-CoA) are involved in signal transduction via myristoylated and palmitoylated proteins, binding to and regulation of transcription factors, and in formation of secretory vesicles.

The fact that there exist three separate classes of lipid-binding proteins which can bind acyl CoA exemplifies the complexity and perhaps functional redundancy often encountered in the regulation of various LCFA-CoA processes within the cell. To date ACBP, which does not appear to bind ligands other than LCFA-CoA, may be specific for its actions involving LCFA-CoA molecules in the cytosol. The fact that the FABP and SCP-2 also bind and modulate LCFA-CoA at first glance may represent overlapping and multiplicity of functions of these proteins. Yet, their differing subcellular distributions argue against all three proteins functioning identically in the cell. In tissues and cells containing ACBP, SCP-2, and FABP, all three protein families may contribute to LCFA-CoA utilization. At the same time the broad ligand specificity of the FABP and SCP-2 allows the regulation of a variety of other cellular processes involving fatty acid metabolism, cholesterol transport, etc. In liver, the concentration of L-FABP is an order of magnitude higher than ACBP. Both proteins can bind LCFA-CoA, but ACBP exhibits a binding affinity one or more orders of magnitude higher than that of L-FABP. Therefore, in liver, ACBP may be more important under conditions of low levels of LCFA-CoA while L-FABP may contribute additional functions under conditions in which LCFA-CoA levels exceed the binding capacity of ACBP. The potential contribution of SCP-2 remains to be determined, but its high concentration in peroxisomes (which apparently do not contain L-FABP and ACBP) suggests an important role of SCP-2 in LCFA-CoA metabolism in this organelle. Future work will also need to focus on investigating the interactions of ACBP, FABP, and SCP-2 with regulatory molecules within cells as well as determining their function both *in vitro* and *in vivo*. Essential will be studies comparing the three LCFA-CoA binding proteins to ascertain their relative contribution in various functions involving LCFA-CoA.

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Quantitative Analysis of Triglycerides Using Atmospheric Pressure Chemical Ionization–Mass Spectrometry

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ABSTRACT: Atmospheric pressure chemical ionization–mass spectrometry (APCI–MS) was used for quantitative analysis of triglycerides (TG) separated by reverse-phase high-performance liquid chromatography. APCI–MS was used for analysis of mono-acid TG standards containing deuterated internal standard, of a synthetic mixture of heterogeneous TG, of randomized and normal soybean oils and of randomized and normal lard samples. Quantitation of the TG by four approaches based on APCI–MS were compared, and these were compared to quantitation obtained using liquid chromatography (LC) with flame-ionization detection (FID). The APCI–MS methods were based on (i) calibration curves from data for mono-acid TG standards, (ii) response factors obtained from a synthetic mixture of TG, (iii) response factors calculated from comparison of randomized samples to their statistically expected compositions, and (iv) response factors calculated from comparison of fatty acid (FA) compositions calculated from TG compositions to FA compositions obtained by calibrated gas chromatography (GC) with FID. Response factors derived from a synthetic mixture were not widely applicable to samples of disparate composition. The TG compositions obtained using APCI–MS data without application of response factors had average relative errors very similar to those obtained using LC–FID. Numerous TG species were identified using LC/APCI–MS which were undetected using LC–FID. Two quantitation methods, based on response factors calculated from randomized samples or on response factors calculated from FA compositions, both gave similar results for all samples. The TG compositions obtained using response factors calculated from FA compositions showed less average relative error than was obtained from LC–FID data, and were in good agreement with predicted compositions for the synthetic mixture and for randomized soybean oil and lard samples.

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Early (1) and recent (2) reviews have summarized the use of several types of two-dimensional detection methods for analysis of triglycerides (TG) using high-performance liquid chromatography (HPLC). These methods have included refractive index, ultraviolet-vis spectrophotometric, moving belt flame-ionization (FID), and evaporative light-scattering (ELSD) detection. While each of these detection methods has its strengths and weaknesses with regard to solvent compatibility, sensitivity, and other factors, the accuracy of the quantitative data of all these methods is dependent on the quality of the chromatographic resolution of the TG species. In complex mixtures which contain numerous species with the same equivalent carbon numbers, complete, or even partial, resolution of chromatographically overlapped species may not be possible. To solve this problem several types of mass spectrometric detection have been successfully applied to the identification and quantitation of molecular species of TG mixtures. Several interface types, such as fast atom bombardment, thermospray, direct inlet, and electrospray have been used, and these applications have been recently reviewed (3). One of the simplest, yet effective, ionization interfaces for use in a chromatographic system has been the direct inlet interface.

We have reported results that demonstrated that atmospheric pressure chemical ionization (APCI) is similarly a simple, versatile ionization source for analysis of TG. Our recent publications (4,5) described the application of APCI–mass spectrometry (MS) to the qualitative analysis of simple homogeneous (mono-acid) TG and genetically modified soybean oils (SBO) separated using reverse-phase HPLC (RP-HPLC). We showed that the mass spectra of TG obtained using APCI–MS were relatively simple and consisted primarily of diglyceride (DG) ions, $[M - RCOO]^+$ or $[DG]^+$, and protonated molecular ions, $[M + 1]^+$. The degree of unsaturation was the primary factor in determining the amount of DG vs. TG ions formed. The mass spectra of TG containing more than four sites of unsaturation contained protonated TG ions as base peaks. DG fragments were the base peaks in spectra of TG containing less than three sites of unsaturation. Mass spectra of TG with three or four sites of unsaturation contained either DG fragments or protonated TG ions as base peaks, depending on the fatty acid (FA) distribution within the TG. No TG protonated molecular ion (only DG) was observable from TG containing only saturated FA. Thus, the utility of APCI–MS for qualitative analysis of TG has been well demonstrated, but the potential for quantitative analysis using this ionization technique has not been explored.

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Abbreviations: ACN, acetonitrile; APCI–MS, atmospheric pressure chemical ionization–mass spectrometry; DG, diglyceride; EIC, extracted ion chromatogram; ELSD, evaporative light-scattering detector; FA, fatty acid; FAME, fatty acid methyl esters; FID, flame-ionization detector; GC, gas chromatography; L, linoleic acid (18:2); LC, liquid chromatography; Ln, linolenic acid (18:3); M, myristic acid (14:0); O, oleic acid (18:1); P, palmitic acid (16:0); Po, palmitoleic acid (16:1); RP-HPLC, reverse-phase high-performance liquid chromatography; S, stearic acid (18:0); SBO, soybean oil; TG, triglyceride.

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Reported here is the extension of APCI–MS to the quantitative analysis of several model and natural TG samples. We report the quantitative analysis of a synthetic mixture containing homogeneous (mono-acid) TG with d_{12}-tripalmitin as internal standard, of a synthetic mixture of thirty-five TG with randomly distributed FA, of randomized and normal SBO and of randomized and normal lard samples. Comparison of quantitative results based on several approaches is presented: (i) construction of calibration curves from mono-acid TG standards, (ii) response factors obtained from a synthetic mixture of heterogeneous (mixed-acid) TG, (iii) response factors calculated from the comparison of randomized samples to their statistically expected compositions, and (iv) response factors calculated from comparison of compositions calculated from TG compositions to compositions obtained by calibrated gas chromatography (GC) with FID detection.

MATERIALS AND METHODS

TG. TG standard mixture "HPLC #G-2" was obtained from Nu-Chek-Prep (Elysian, MN). The TG standard mixture contained equal weights of the following mono-acid TG: tripalmitin (PPP), tripalmitolein (PoPoPo), tristearin (SSS), triolein (OOO), trilinolein (LLL), and trilinolenin (LnLnLn), where P is palmitic acid, S is stearic acid, O is oleic acid, L is linoleic acid, and Ln is linolenic acid. A stock solution of "HPLC #G-2" was used to prepare solutions containing: 30 μg/μL, 15 μg/μL, 6 μg/μL, 3 μg/μL, 0.6 μg/mL, 0.3 μg/μL, and 0.06 μg/μL total TG. This resulted in solutions containing the following amounts of individual TG standards: 5 μg/μL, 2.5 μg/μL, 1.0 μg/μL, 0.5 μg/μL, 0.1 μg/μL, 0.05 μg/μL, and 0.01 μg/μL. d_{12}-PPP was added to each standard solution at a concentration of 1.01 μg/μL as internal standard. The d_{12}-PPP was prepared in our laboratory by reduction of methyl *cis*-9-hexadecenoate-13,13,14,14-d_4 [prepared in a similar manner as the methyl 9-*cis*-octadecenoate analogue (6)] with Wilkinson's Catalyst (7) and hydrogen to the saturated fatty acid methyl ester (FAME), methyl palmitate-d_4. The FAME was converted to the FA with alcoholic KOH and reacted with glycerol and *p*-toluenesulfonic acid to yield d_{12}-PPP (8).

Synthetic mixture of TG from FA. FA (all >99% pure) were purchased from Nu-Chek-Prep. Five FA were used: P, S, O, L, and Ln. The mixed FA TG standard was prepared by the *p*-toluenesulfonic acid-catalyzed esterification of glycerol with an equimolar mixture of the FA (8). Glycerol and *p*-toluenesulfonic acid (Fisher Scientific Co., Fair Lawn, NJ) were used as received. All solvents were HPLC quality and were used without further purification.

Natural samples. Refined, bleached, and deodorized SBO was obtained from PVO Foods, Inc. (St. Louis, MO) and was randomized using the sodium-catalyzed interesterification method of List *et al.* (9). Refined and deodorized native lard was obtained from Colfax (Pawtucket, RI.) The lard was randomized by Nabisco Foods (Indianapolis, IN) using a similar method for randomization (interesterification).

GC. FAME were analyzed using a Varian 3400 (Palo Alto, CA) gas chromatograph equipped with a Supelco (Belefonte Park, PA) SP2380 30 m × 0.25 mm i.d. capillary column. The conditions were: inlet temperature = 240°C; detector temperature = 280°C; initial temperature = 150°C; initial time = 35 min; ramp to 210°C at 3°C/min. Methyl esters were made by the sodium methoxide esterification according to the method of Glass (10).

Liquid chromatography (LC). Solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI) or EM Science (Gibbstown, NJ). Solvents were HPLC grade or the highest available quality and were used without further purification. The HPLC pump used for LC/APCI–MS was an LDC 4100 MS (Thermo Separation Products, Schaumburg, IL) quaternary pump system with membrane degasser. The columns used were an Adsorbosphere C18 (Alltech Associates, Deerfield, IL), 25 cm × 4.6 mm, 5 μm (12% carbon load) in series with an Adsorbosphere UHS C18 25 cm × 4.6 mm, 10 μm (30% carbon load). The flow rate throughout was 1 mL/min. A gradient solvent program with propionitrile (PrCN), dichloromethane (DCM), and acetonitrile (ACN) was used to separate the mixture of homogeneous TG containing d_{12}-tripalmitin as internal standard. The gradient used was as follows: initial—PrCN/DCM/ACN (45:20:35, by vol); linear from 15 to 20 min to PrCN/DCM/ACN (45:25:30, by vol), held until 35 min; linear from 35 to 40 min to PrCN/DCM/ACN (45:30:25, by vol), held until 95 min. Separations incorporating PrCN provided slightly better peak shapes than those without, but due to variations in lot-to-lot solvent quality, the change was made to a DCM/ACN system. APCI source temperature and gas flows were optimized to minimize the amount of undesirable adduct formation.

Separations of the synthetic randomized mixture of heterogeneous TG and of the randomized and normal SBO and lard samples were accomplished using gradient elution as follows: initial—ACN/DCM (65:35, vol/vol); linear from 20 to 25 min to ACN/DCM (60:40, vol/vol), held until 35 min; linear from 35 to 40 min to ACN/DCM (55:45, vol/vol), held until 50 min; linear from 50 to 60 min to ACN/DCM (55:45, vol/vol), held until 85 min 5 μL of each sample solution was injected. The column effluent was split so that ~850 mL/min went to an ELSD and ~150 μL/min went to the APCI interface. ELSD data were used only to confirm proper operation of the LC system; these data are not presented. The ELSD was an ELSD MKIII (Varex, Burtonsville, MD). The drift tube was set at 140°C, the gas flow was 2.0 standard liters per minute. High purity N_2 was used as the nebulizer gas.

The quantitative analysis by RP-HPLC with FID detection used a linear gradient of ACN/DCM 70:30 to 40:60 vol/vol over 120 min. Columns and conditions were as previously described (11). The gradient used for LC–FID could not be used for APCI–MS analysis because the mass spectrometer's LC software did not allow gradient runs over 99 min in length.

MS. A Finnigan MAT (San Jose, CA) SSQ 710C mass spectrometer fitted with an APCI source was used to acquire mass spectral data. The vaporizer was operated at 400°C, and

the capillary heater was operated at 265°C. The corona voltage was set at 6.0 μA throughout. Auxiliary and sheath gases were set to 35 psi and 5 mL/min, respectively. Spectra were obtained from *m/z* 400 to *m/z* 1000 or *m/z* 1100, with a scan time of 1.75 to 2.0 s. Preliminary data showed no substantial ions below *m/z* 400. Chromatograms were processed using three-point smoothing for graphical output, but no smoothing was applied during quantitation of extracted ion chromatograms. All mass spectra shown represent an average of spectra over the breadth of a chromatographic peak. Nominal masses, shown in mass spectra, were obtained by application of a mass defect of 0 mmu @ 0 amu to 700 mmu @ 1000 amu.

Lipase hydrolysis. Positional isomers were determined using lipase hydrolysis as previously described (12). Briefly, lipase (EC 3.1.1.3) was used to cleave the FA at glycerol 1,3 carbons, and the lipolysis products were separated using solid phase extraction. The FAME of the lipolysis products were analyzed using GC–FID, and the composition obtained was compared to the FAME composition obtained for the intact samples.

Calculations. The expected composition of the synthetic mixture was calculated from the FA composition. If FA are esterified with glycerol, the number of possible TG molecular species, excluding isomers, can be calculated from the equation (13):

$$\text{number of TG molecular species} = \frac{n^3 + 3n^2 + 2n}{6} \quad [1]$$

where n is the number of FA. When 5 FA are used, 35 TG molecular species are possible. The 5 FA would be randomly distributed between all possible molecular species. Formulas for calculating the composition of a randomly distributed mixture of TG were derived from probability theory (14). The following equations were used to calculate the percentages of TG containing one (Eq. 2), two (Eq. 3), or three (Eq. 4) different FA:

$$\text{TAG\%} = \frac{\text{FA\%} \cdot \text{FA\%} \cdot \text{FA\%}}{10{,}000} \quad [2]$$

$$\text{TAG\%} = \frac{(\text{FA1\%} \cdot \text{FA1\%} \cdot \text{FA2\%}) \cdot 3}{10{,}000} \quad [3]$$

$$\text{TAG\%} = \frac{(\text{FA1\%} \cdot \text{FA2\%} \cdot \text{FA3\%}) \cdot 6}{10{,}000} \quad [4]$$

These equations also were used to calculate the theoretical compositions of the randomized SBO and lard samples from their FA compositions determined by calibrated GC–FID.

The percent relative errors for TG were calculated by subtracting the statistically expected amount from the determined amount, multiplying by 100, and then dividing by the statistically expected amount. The percent relative errors for the most abundant TG in a sample were averaged to give the average relative error. TG present at low levels had higher percent relative errors, so these were not included in calculation of the average relative error for a sample. Similarly, the percent relative errors for the FA was calculated by comparison of the FA composition calculated from the TG composition to the FA composition determined by calibrated GC–FID. The individual FA relative errors were averaged to give the average relative error for the FA composition.

RESULTS

Homogeneous mixture. Seven concentrations of a six-component homogeneous TG standard mixture were prepared, and raw quantitative data were obtained by summing the areas under the $[DG]^+$ and $[M + 1]^+$ peaks. The total areas under the $[DG]^+$ and $[M + 1]^+$ peaks were obtained from extracted ion chromatograms (EIC), in which a chromatogram was produced by selectively extracting the appropriate $[DG]^+$ and $[M + 1]^+$ *m/z* for each TG from the total, or reconstructed ion chromatogram.

Calibration curves were constructed by referencing the total peak areas for each TG to the area obtained for the deuterated d_{12}-PPP internal standard $[d_8\text{-DG}]^+$ peak having *m/z* = 559.5. Example calibration curves for three of the homogeneous TG standards are shown in Figure 1. The sensitivities (line slopes) and intercepts are given in slope/intercept form for the six components as follows: LnLnLn $y = 0.1201\,x + 0.05481$; LLL $y = 0.1199\,x + 0.05297$; OOO $y = 0.1471\,x + 0.05605$; SSS $y = 0.1337\,x + 0.06286$; PoPoPo $y = 0.2382\,x + 0.04958$; PPP $y = 0.1957\,x + 0.04113$, where y is the ratio of the area of the analyte peak to the area of the internal standard peak and x is the amount, in μg, actually injected. The correlation coefficients were LnLnLn =.9986, LLL = .9964, OOO = .9993, SSS = .9966, PoPoPo = .9987, PPP = .9998. As expected, the fit of the calibration curves was improved by decreasing the concentration ranges in the calibration curves. If only the lower four concentrations were used (0.01 μg/μL, 0.05 μg/μL, 0.10 μg/μL, and 0.50 μg/μL), the correlation coefficients became: LnLnLn = .9992; LLL = .9999; OOO = 1.0000; SSS = .9999; PoPoPo = 1.0000; PPP = 1.0000. PPP and PoPoPo had correlation coefficients of 1.0000 if the point obtained for 1.0 μg/μL was also included.

Of the 0.01 μg/μL solution, 5 μL contained 50 ng of each TG. This amount was the approximate detection limit of tristearin under these chromatographic conditions. The detection limit of tripalmitin was lower. Use of a micro flow chromatographic system with no splitting of the effluent stream would likely result in lower limits of detection.

35-Component synthetic TG mixture. An FID chromatogram and an annotated MS total ion chromatogram of the synthetic mixture of heterogeneous TG are shown in Figure 2. Although different gradients of ACN/DCM were used on the two systems, the chromatograms gave similar peak patterns. The shorter gradient run on the LC/APCI–MS system produced less resolution than the longer gradient used for the LC–FID run. The heated capillary interface between the atmospheric pressure and high vacuum regions of the ionization interface also resulted in a slight loss in peak resolution. In spite of higher resolution in the FID chromatogram, several peaks were present which contained unresolved overlapped TG. The TG which were unresolved in

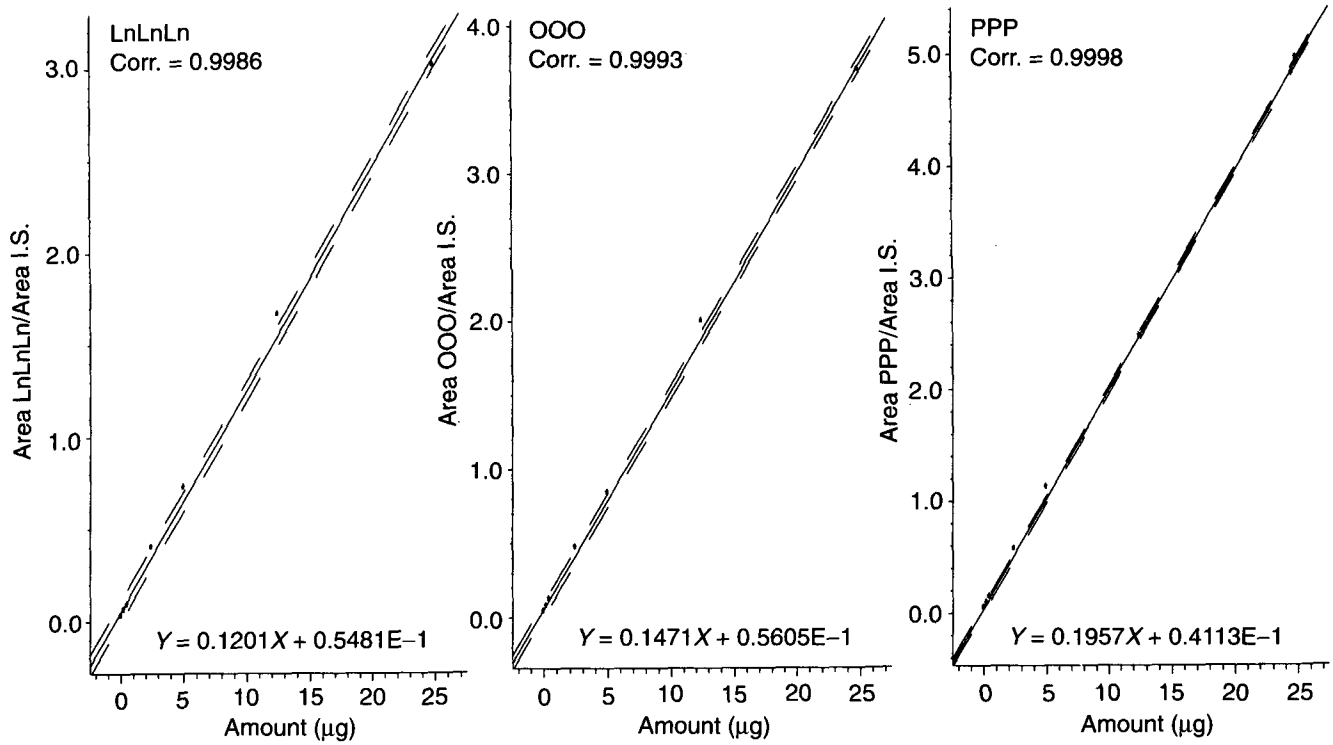


FIG. 1. Calibration curves for LnLnLn, OOO, and PPP where Ln is linolenic acid, O is oleic acid, and P is palmitic acid. Plots represent the total area for each triglyceride divided by the area of the internal standard peak, d_{12}-PPP, vs. the amount of each triglyceride injected. Line equations are given in slope/intercept form.

FID chromatograms were readily resolved by their different *m/z* in EIC. Because each TG produced up to three DG fragments, unresolved peaks in one EIC were resolved in other EIC of $[DG]^+$ arising from the same TG. The appearance of peaks in several EIC which all arose from one TG greatly facilitated the qualitative identification of individual TG in complex mixtures.

EIC of $[DG]^+$ *m/z* are shown in Figure 3. Each EIC showed an elution pattern which was based on the equivalent carbon number of the fatty acyl chains attached to the DG. The areas under the peaks in the $[DG]^+$ EIC were summed with the areas under peaks in EIC for $[M + 1]^+$ ions to provide a total area for each TG. The composition calculated from the combination of "raw" $[DG]^+$ and $[M + 1]^+$ ion areas for each TG in the EIC of the APCI–MS data is shown in Table 1. The composition determined by LC with FID detection is also given in Table 1. Positional isomers of the TG were not distinguished, so the abbreviations given in Table 1 identify molecular species without respect to the positional arrangement of the FA. Peaks in the EIC of *m/z* 603.5, arising from $[OO]^+$ and $[SL]^+$, were only partially resolved, so they were manually integrated to give the best approximation of appropriate peak areas. This partial overlap resulted in some variability, however, as demonstrated by the largest standard deviation in Table 1 shown by OLS. In the EIC of *m/z* 601.5, an unresolved overlap occurred between the "P" peak of $[OL]^+$ (#1) and the "O" peak of $[SLn]^+$ (#2). The ratios of the "Ln_1," "L_1," and "S_1" peaks to the "Ln_2," "L_2," and "S_2" peaks were used to apportion the overlapped peak, so that O_1/O_2 and P_1/P_2 were in the same proportion as $[(Ln_1 + L_1 + S_1)/(Ln_2 + L_2 + S_2)]$. Again, however, these individual peak areas represent only one of several peaks which contribute to the total area for a given TG molecular species.

The FA composition of the heterogeneous calibration mixture was determined (as the FAME) by GC with FID. The FAME mole percent composition was Ln = 18.5%; L = 19.8%; O = 20.2%; S = 20.6%; P = 20.9%. Using the above equations to calculate the randomly distributed TG percentage composition gave the statistical percentage composition shown in Table 1. The data in Table 1 demonstrate that the APCI–MS data require response factors to adjust the raw percentages to agree with the expected percentages. The areas under peaks attributable to the TG species (and the percentage composition) were dependent on the acyl chain length and on the fragmentation behavior of the TG, which was dependent on the number of sites of unsaturation in the TG species. Trisaturated TG produced virtually only $[DG]^+$ ion, with no $[M + 1]^+$ ion observed. Since the $[DG]^+$ ions, having lower *m/z*, were propagated through the MS system more efficiently than larger ions, they gave larger response than was observed for those TG which gave significant $[M + 1]^+$ ions. Conversely, TG containing polyunsaturated fatty acyl chains gave $[M + 1]^+$ ions as base peaks and showed less signal response. Other methods of mass spectrometric quantitation of TG have reported similar effects from unsaturation and/or chain length (15–19). Thus, Table 1 indicates that SSS was overrepresented, or gave a percentage composition larger than

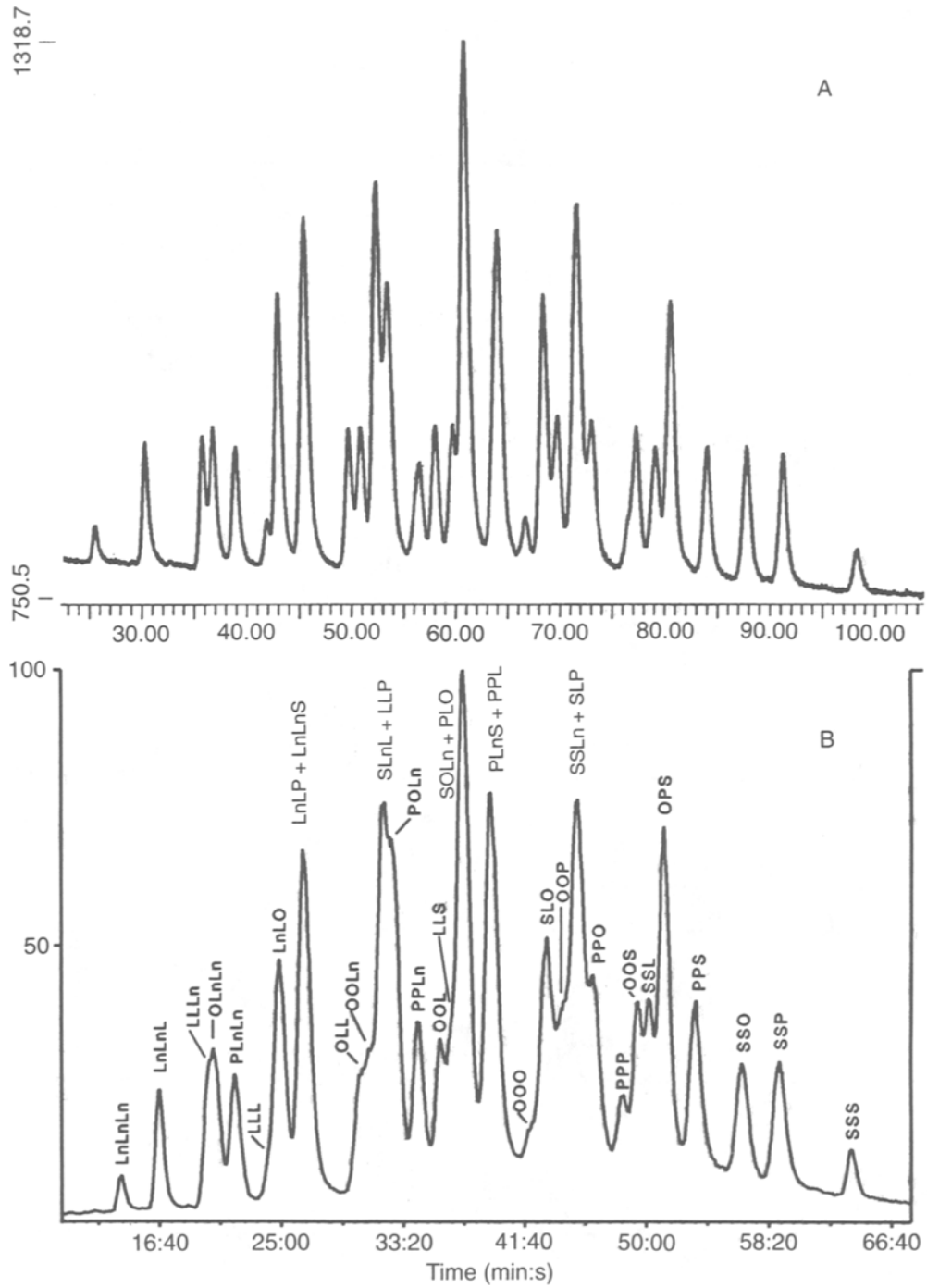


FIG. 2. A) Liquid chromatography–flame-ionization detection and B) liquid chromatography/atmospheric pressure chemical ionization–mass spectrometry chromatograms of a synthetic mixture of 35 triglycerides containing five randomly distributed fatty acids. Peak labels identify individual triglyceride molecular species. See Figure 1 for abbreviations; S, stearic acid; L, linoleic acid.

the calculated amount, while LnLnLn was underrepresented, or gave a percentage composition less than the calculated amount. PPP was overrepresented the most, as it had shorter acyl chains in addition to saturated acyl chains. Though not directly correlated, the same trend in dependence of the quantitative peak areas on the qualitative appearance of the spectra was seen in the sensitivities (slopes) of the calibration curves presented above. The sensitivity of the PPP calibration curve was larger than that for the SSS calibration curve, which was larger than that for the LnLnLn calibration curve. However, TG containing monounsaturated fatty acyl chains gave higher sensitivities than tri-saturates in the calibration curves.

Example mass spectra of four TG species are shown in

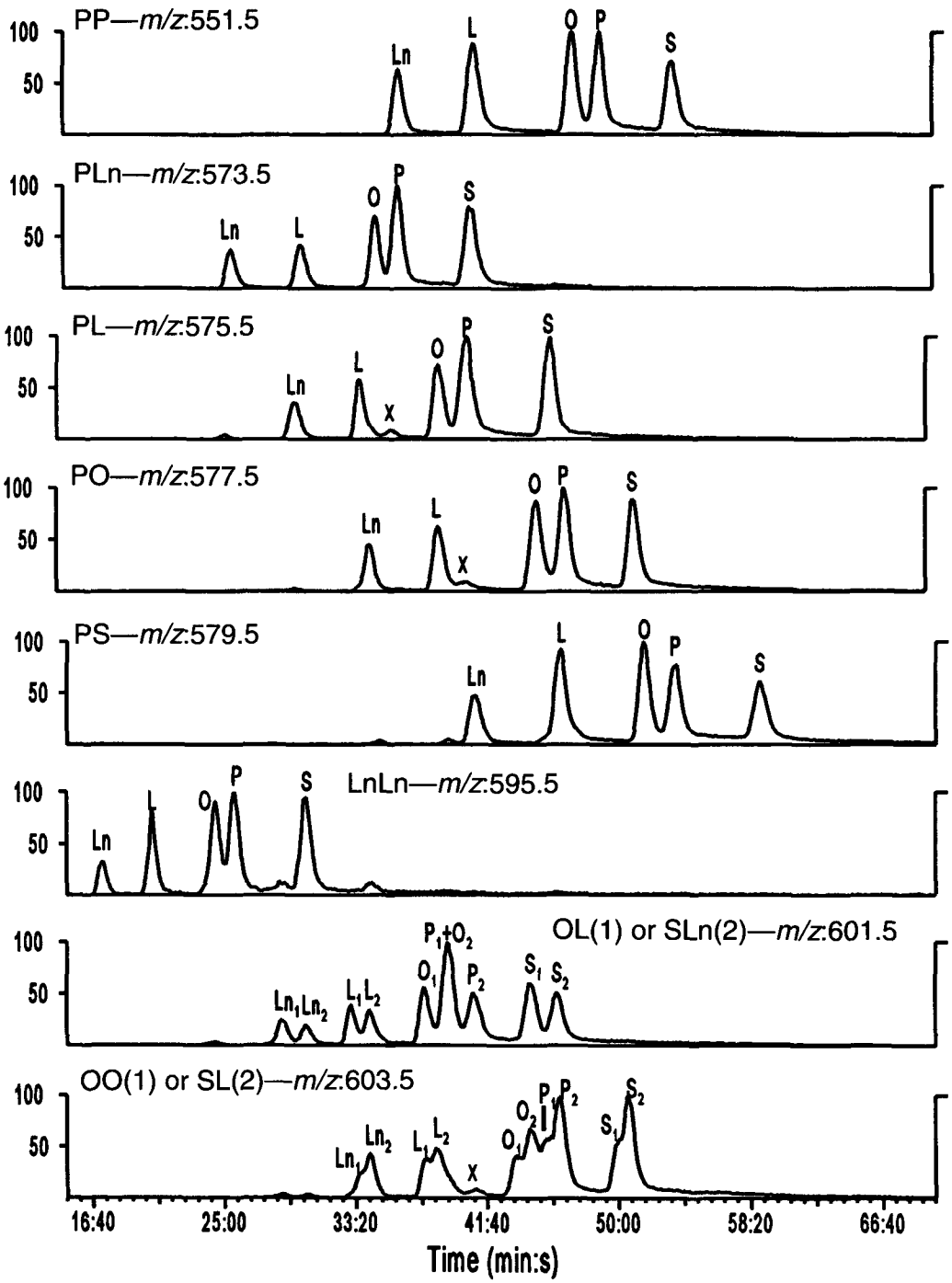


FIG. 3. Extracted ion chromatograms of [diglyceride (DG)]$^+$ *m/z* from a synthetic mixture of 35 triglycerides. [DG]$^+$ identity and *m/z* are given in the upper left of each chromatogram. The peaks are labeled with the identity of the fatty acid which, in combination with the [DG]$^+$, make each of the triglyceride molecular species. See Figures 1 and 2 for other abbreviations.

Figure 4. These spectra demonstrate that, as previously reported, spectra of TG containing more than four sites of unsaturation had [M + 1]$^+$ ions as base peaks, while spectra of TG containing less than three sites of unsaturation had [DG]$^+$ ions as base peaks. Though conditions were optimized to minimize the amount of PrCN adducts formed, these spectra showed distinct PrCN adduct ions. The separations carried out using only ACN/DCM gradients resulted in spectra containing virtually no adduct ions.

One method to produce response factors was to calculate a set of empirical factors for each TG which, when multiplied by the raw MS composition, gave the statistical composition.

TABLE 1
Percentage Composition of Heterogeneous TG Synthetic Mixture

TG[a]	Average raw MS % (n = 3)[b]	Statistical % from GC–FID[c]	GC–FID RF adjusted %[d]	Average LC–FID % (n = 3)
POS	6.3 ± 0.5	5.2	5.5	5.7 ± 0.2
PLS	5.8 ± 0.2	5.1	5.0	6.3 ± 0.2
PLO	4.5 ± 0.2	5.0	4.4	6.9 ± 0.3
LOS	4.4 ± 0.7	5.0	5.0	4.9 ± 0.2
PLnS	4.7 ± 0.3	4.8	4.7	4.1 ± 0.1
PLnO	4.5 ± 0.3	4.7	4.3	5.5 ± 0.2
LnOS	4.1 ± 0.3	4.6	4.3	4.3 ± 0.2
PLnL	4.5 ± 0.2	4.6	4.8	5.5 ± 0.2
LnLS	3.7 ± 0.1	4.5	4.1	3.8 ± 0.1
LnLO	3.8 ± 0.4	4.4	4.9	4.8 ± 0.3
PPS	3.6 ± 0.5	2.7	2.8	2.3 ± 0.2
SSP	3.0 ± 0.3	2.7	2.5	2.3 ± 0.3
PPO	3.7 ± 0.3	2.6	2.7	2.9 ± 0.1
PPL	3.1 ± 0.2	2.6	2.5	3.3 ± 0.1
SSO	2.9 ± 0.3	2.6	2.6	2.6 ± 0.1
OOP	2.9 ± 0.3	2.6	2.5	2.7 ± 0.0
SSL	2.8 ± 0.2	2.5	2.8	2.3 ± 0.1
OOS	2.5 ± 0.0	2.5	2.7	2.6 ± 0.1
LLP	2.4 ± 0.1	2.5	2.3	3.1 ± 0.1
LLS	2.2 ± 0.0	2.4	2.3	1.7 ± 0.1
OOL	2.5 ± 0.1	2.4	2.7	2.0 ± 0.2
PPLn	3.1 ± 0.2	2.4	2.5	1.8 ± 0.1
LLO	2.3 ± 0.1	2.4	2.8	1.9 ± 0.0
SSLn	2.3 ± 0.2	2.4	2.4	1.9 ± 0.1
OOLn	1.7 ± 0.1	2.3	2.0	2.1 ± 0.1
LLLn	1.9 ± 0.2	2.2	2.5	1.8 ± 0.1
LnLnP	1.9 ± 0.1	2.1	2.2	2.1 ± 0.1
LnLnS	1.8 ± 0.1	2.1	2.4	1.7 ± 0.1
LnLnO	1.5 ± 0.0	2.1	1.9	2.4 ± 0.1
LnLnL	1.4 ± 0.2	2.0	2.0	1.7 ± 0.2
PPP	1.5 ± 0.1	0.9	1.0	0.5 ± 0.1
SSS	1.0 ± 0.2	0.9	0.8	0.8 ± 0.1
OOO	0.7 ± 0.2	0.8	0.8	0.5 ± 0.0
LLL	0.5 ± 0.1	0.8	0.6	0.5 ± 0.1
LnLnLn	0.4 ± 0.0	0.6	0.6	0.5 ± 0.0
Sum	100.0	100.0	100.0	100.0
Avg. relative error %	17.0	—	7.4	17.4

[a]Abbreviations: TG, triglyceride; P, palmitic acid; Ln, linolenic acid; L, linoleic acid; O, oleic acid; S, stearic acid; RF, response factor. TG names do not indicate positional location of fatty acids (FA) in TG.
[b]TG composition obtained from the sum of areas under diglyceride and $[TG + 1]^+$ peaks in extracted ion chromatograms from liquid chromatography (LC)/atmospheric pressure chemical ionization–mass spectrometry (MS) data.
[c]TG composition calculated from the FA composition determined by gas chromatography–flame-ionization dection (GC–FID), assuming a random distribution of FA.
[d]TG composition obtained by multiplying raw composition by response factor calculated in two steps: (i) response factor for each FA was calculated by dividing the FA percentage determined by GC–FID by the FA percentage calculated from the raw TG composition determined by LC/MS; (ii) response factors for each TG were obtained by multiplying together FA response factors for each FA in the TG.

TABLE 2
Fatty Acid Percentage Composition of Heterogeneous TG Synthetic Mixture[a]

FA	From raw MS TG[b]	From calibrated GC–FID	From GC–FID-adjusted TG[c]	From LC–FID TG[d]
P	24.0	20.9	20.9	22.1
Ln	16.3	18.5	18.2	17.6
L	18.5	19.8	19.2	20.0
O	19.8	20.2	20.7	20.8
S	21.4	20.6	20.9	19.4
Sum	100.0	100.0	100.0	100.0
Avg. relative error %	8.0	—	1.7	4.0

[a]Abbreviations as in Table 1.
[b]FA composition calculated from the TG composition determined by LC/MS from the sum of $[DG]^+$ and $[TG + 1]^+$ peaks.
[c]FA composition calculated from the adjusted TG composition which was obtained by application of response factors derived from the ratio of FA compositions of the raw MS data and GC–FID data.
[d]FA composition calculated from the TG composition determined using LC with FID detection.

This set of response factors for each TG could then be applied to other TG mixtures containing a similar set of TG. Response factors obtained from the synthetic mixture were applied to the randomized SO sample presented below.

A second method for producing response factors was to compare the FA composition calculated from the TG composition to the FA composition determined by calibrated GC–FID. The FA compositions determined by GC–FID and calculated from the "raw" MS data are given in Table 2. The amount of palmitic acid calculated from the TG composition was larger than the amount determined by GC–FID, as was the amount of stearic acid. The amount of linolenic acid calculated from the TG composition was less than that determined by GC–FID. Response factors for each FA were calculated by dividing the FA composition determined by calibrated GC with FID detection by the FA composition calculated from the TG composition given from the "raw" APCI–MS data. These numbers were then normalized to the smallest value to produce a set of factors: P = 1.00; S = 1.12; O = 1.20; L = 1.19; Ln = 1.29. Response factors for each TG were calculated by simply multiplying the appropriate FA response factors together. For example, the response factor for PPP was $1.00 \cdot 1.00 \cdot 1.00 = 1.00$, while that for LLL was $1.19 \cdot 1.19 \cdot 1.19 = 1.68$. Application of the response factors calculated from the GC–FID FA composition gave the TG composition listed in Table 1.

It can be seen that the TG composition adjusted using the GC–FID-derived response factors agrees more closely with the calculated composition than does the composition determined by LC–FID, which is accepted for use for quantitation without response factors (20). The average relative error for the GC–FID-adjusted APCI–MS TG composition was 7.4% per TG compared to the predicted composition. The average relative error of the TG composition determined by LC–FID was 17.4% per TG compared to the predicted composition. The composition determined by LC–FID had an average relative error very similar in magnitude to the "raw" APCI–MS composition. The FA composition resultant from the response-factor-adjusted APCI–MS TG composition, Table 2, had an average relative error of 1.7% per FA compared to the FA composition determined by GC–FID. The FA composi-

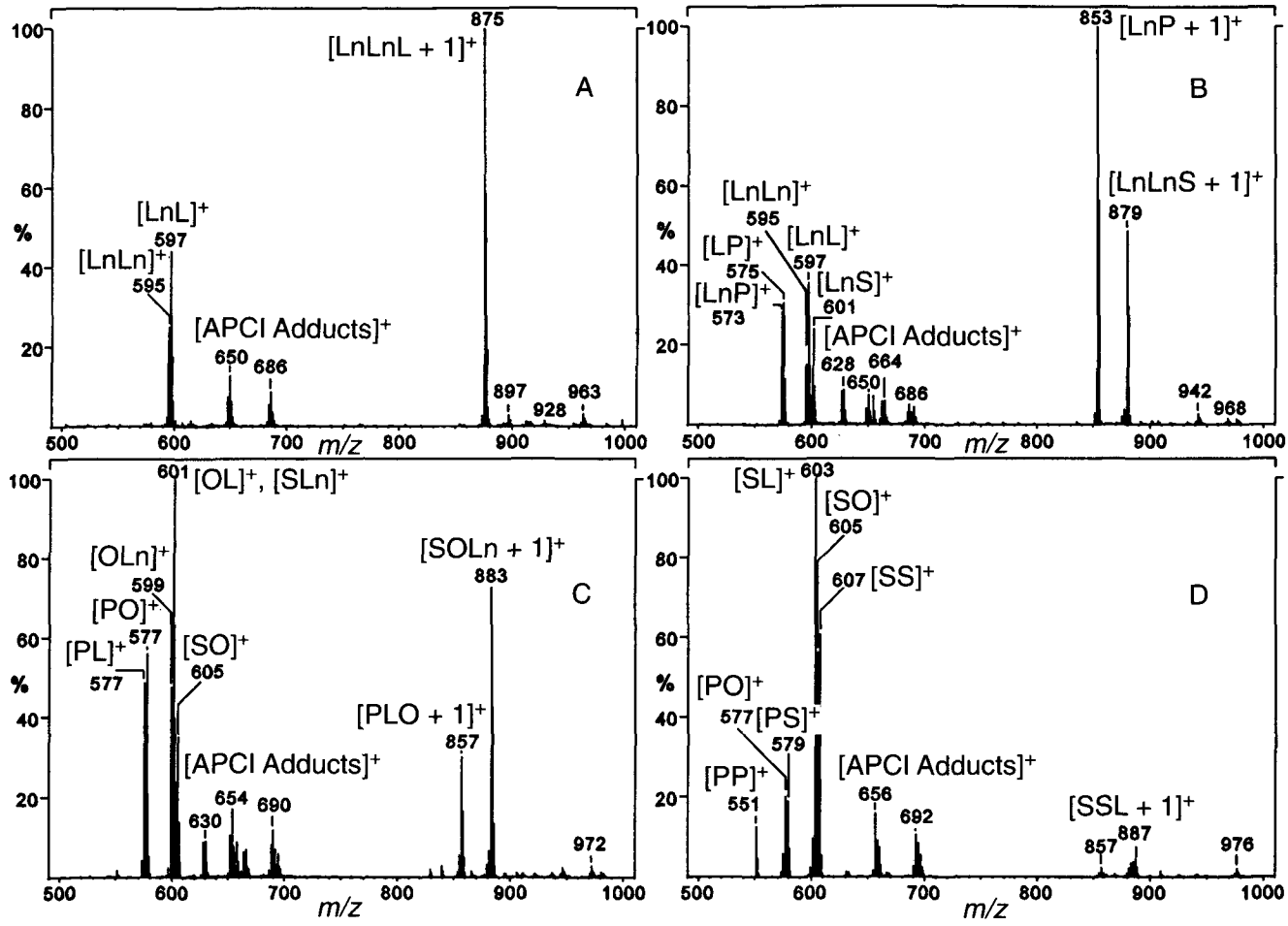


FIG. 4. Mass spectra of A) LnLnL (8 sites), B) LnLP and LnLnS (5 and 6 sites), C) PLO and SOLn (3 and 4 sites), and D) SSL with smaller amounts of OOS and OPS (2, 1 sites). Adducts formed were the $[DG + 53]^+$ and $[DG + 89]^+$ ions derived from addition of propionitrile [molecular weight (MW) = 55 amu)] and from addition of propionitrile plus acetonitrile (MW = 38 amu). See Figures 1, 2, and 3 for abbreviations; APC1, atmospheric pressure–chemical ionization.

tion resultant from the TG composition obtained from the LC–FID data had an average relative error of 4.0% per FA.

Randomized SBO. Chromatograms of randomized SBO obtained using LC–FID and LC/APCI–MS are shown in Figure 5. These two methods gave very similar peak patterns and comparable visible resolution. EIC were used to quantitate the $[DG]^+$ and $[M + 1]^+$ peak areas as described above. Example EIC are shown in Figure 6. Example mass spectra are shown in Figure 7. Because the chromatographic separation used an ACN/DCM gradient, these spectra did not show significant adduct formation. Only small amounts of ACN adducts were formed. The "raw" TG composition calculated from the sum of $[DG]^+$ and $[M + 1]^+$ peaks is given in Table 3, as is the composition determined by LC–FID. The theoretical TG composition of the randomized SBO was calculated using the FA composition determined by calibrated GC–FID, given in Table 4. The calculated TG composition is given in Table 3. The FA composition at carbon 2 of the TG determined by GC–FID of the products of lipase hydrolysis is given in Table 4, and may be compared to the composition at carbon 2 of normal SBO. The change in composition at carbon 2 toward that of the total FA composition indicated that randomization was mostly, but not absolutely, complete.

Response factors were produced by comparison of the FA composition calculated from the "raw" LC/APCI–MS data to the FA composition determined by GC–FID. The normalized response factors for the FA were as follows: P = 1.13; S = 1.00; O = 1.36; L = 1.87; Ln = 1.07. As above, these FA response factors were multiplied together to give TG response factors for each of the TG molecular species. The adjusted TG composition obtained using the FA-derived response factors is given in Table 3. These response factors generally reflected the qualitative fragmentation behavior discussed above except for the response factor for linolenic acid. Based on the considerations mentioned above, it was expected that this FA would be underrepresented and require a response factor greater than those for oleic and linoleic acids. In this SBO sample, in contrast to the synthetic mixture, linoleic acid

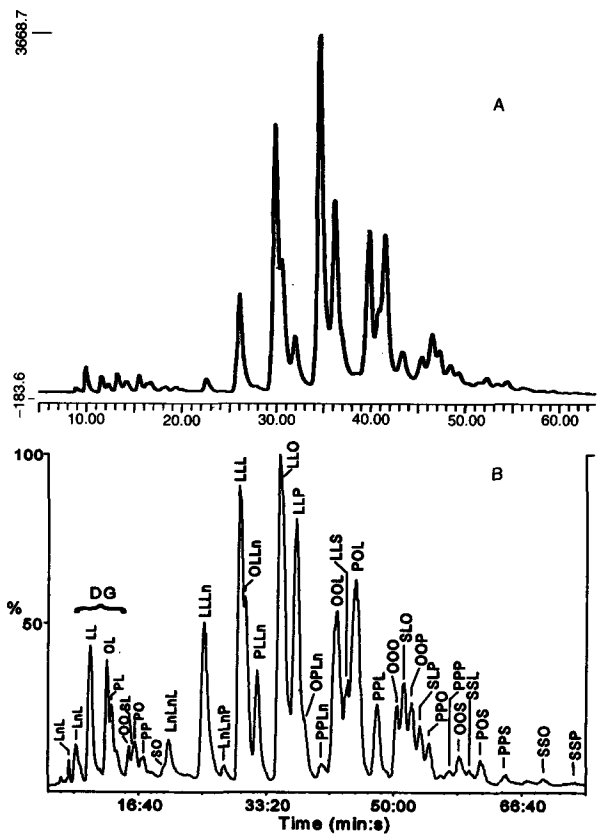


FIG. 5. A) Liquid chromatography–flame-ionization detection and B) liquid chromatography/atmospheric pressure chemical ionization–mass spectrometry chromatograms of randomized soybean oil. Peak labels identify individual DG and triglyceride molecular species. DG, formed during the randomization, eluted first. See Figures 1, 2, and 3 for abbreviations.

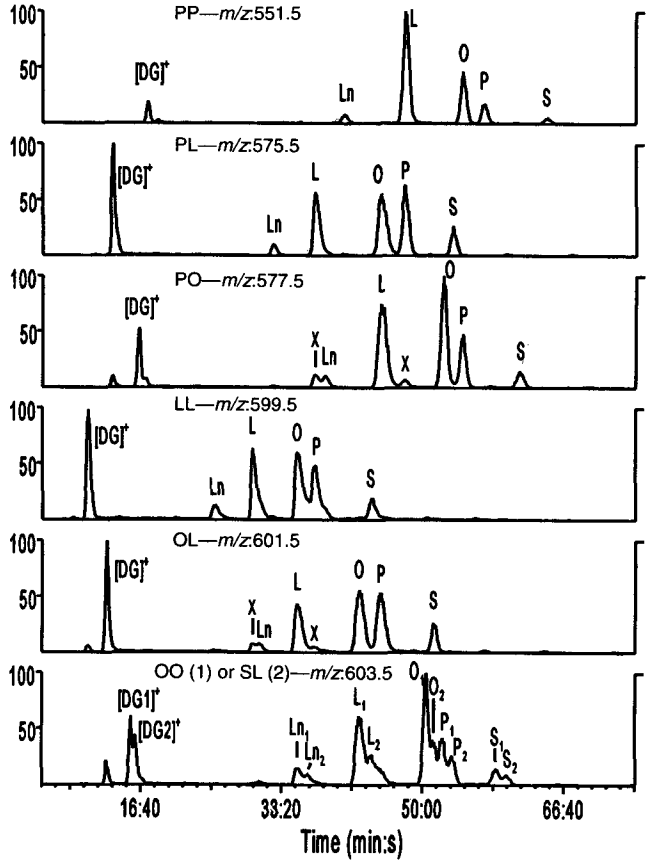


FIG. 6. Extracted ion chromatograms of $[DG]^+$ *m/z* from randomized soybean oil. $[DG]^+$ identity and *m/z* are given in the upper left of each chromatogram. The peaks are labeled with the identity of the fatty acid which, in combination with the $[DG]^+$, make each of the triglyceride molecular species. See Figures 1, 2, and 3 for abbreviations.

was present in a much larger percentage than was linolenic acid. Because of the elution patterns in the EIC, the "L" peak in one EIC gave a ^{13}C isotope peak, for molecules containing two ^{13}C atoms, at nearly the same chromatographic position as the "Ln" peak in the EIC for a DG having one less site of unsaturation (for instance, the "L" peak of the $[PL]^+$ EIC at *m/z* 575.5 gave an isotope peak at the same location as the "Ln" peak in the *m/z* 577.5 EIC of $[PO]^+$). The molecules which contained two ^{13}C isotopes therefore contributed peak area to the peaks in the EIC of the DG having one less site of unsaturation. When linoleic acid was present in a much larger amount than linolenic acid, the contribution of peak area from the isotope-containing molecules caused overestimation of the amount of linolenic acid-containing species. Imperfect chromatographic overlap of the peaks and some small variability in isotope ratios made subtraction of a calculated isotope area impractical. The response factors calculated from the FA composition or from the statistical composition effectively compensated for this overestimation.

The adjusted TG composition obtained using FA-derived response factors was in fair agreement with the composition calculated for a random distribution of the FA. The average relative error for the twenty most abundant molecular species was 8.6% per TG for the TG composition obtained from the GC-adjusted APCI–MS data, compared to the composition expected from a random distribution. The average relative error for the most abundant TG molecular species was 23.9% for the TG composition obtained by LC with FID detection, compared to the TG composition expected from a random distribution. TG molecular species which were not detected using LC–FID, which had relative errors of 100%, were not included in the calculation of the average percent relative error. The FA compositions calculated from the TG compositions obtained from the GC-adjusted MS data and the LC–FID data are given in Table 4. The FA composition calculated from the TG composition obtained from the GC-adjusted MS data had an average relative error of 2.2% per FA compared to the FA composition obtained by GC–FID. The FA composition calculated from the TG composition obtained by LC–FID had an average relative error of 6.5% per FA.

Quantitation based on response factors calculated from the synthetic mixture was also performed. Relative response factors for each TG were calculated by dividing the percentage expected from the random distribution of FA by the TG per-

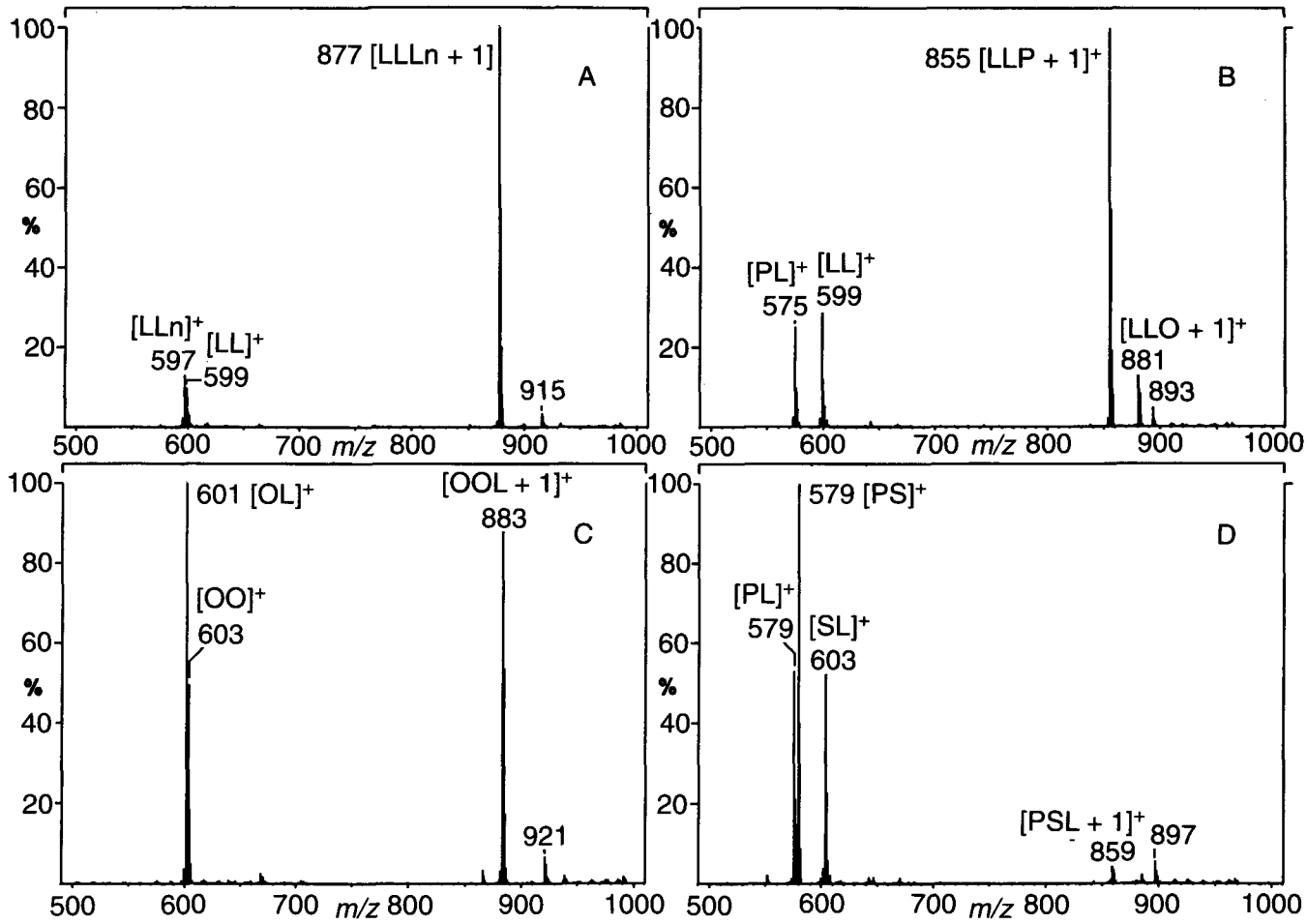


FIG. 7. Mass spectra of A) LLLn (7 sites), B) LLP with a small amount of LLO (4 sites), C) OOL (4 sites), and D) PSL (2 sites). Small amounts of [DG + 38]$^+$ adduct ions derived from addition of acetonitrile (MW = 38 amu) were formed. See Figures 1, 2, and 3 for abbreviations.

centage obtained by APCI–MS. Application of response factors thus obtained to the randomized SBO resulted in the TG composition given in Table 3. The poor agreement of the calculated composition with the expected composition indicated that relative response factors calculated from a sample with a significantly different percentage composition were not useful. The concentration dependence of the relative response factors necessitated use of response factors derived from mixtures of similar composition. The FA composition calculated from the TG composition obtained using response factors obtained from the synthetic mixture had an average relative of 26.7% per FA. Because of the demonstrated lack of utility, the use of response factors calculated from the synthetic mixture was not pursued further.

Normal SBO. A chromatogram obtained using LC with APCI–MS detection is shown in Figure 8. The TG composition obtained from this chromatogram is given in Table 5, listed as "raw" APCI–MS data. The TG composition determined by LC–FID is also given in Table 5. Quantitation using response factors derived from the FA composition determined by GC–FID was performed as described above. The FA composition calculated from the "raw" MS data and the FA composition determined by GC–FID are given in Table 6. The response factors calculated by comparison of the MS to the GC–FID data were: P = 1.12; S = 1.12; O = 1.44; L = 2.03; Ln = 1.00. Response factors for individual TG molecular species were calculated as described above. The TG composition which resulted from application of the response factors thus derived is given in Table 5, referred to as "GC–FID RF Adjusted %."

The FA composition obtained from the TG composition calculated using response factors derived from GC–FID data is given in Table 6. This FA composition showed an average relative error of 4.5% per FA, compared to 19.8% average relative error from the "raw" APCI–MS data and 9.2% average relative error per FA from the LC–FID data.

Quantitation was also performed using response factors calculated from the randomized SBO. Response factors for each of the TG molecular species were calculated by dividing the statistically expected composition for the randomized

TABLE 3
Percentage Composition of Randomized Soybean Oil TG[a]

TG	Raw MS %	Statistical % from GC–FID	GC–FID RF adjusted %	LC–FID %	Mixture adjusted %[b]
LLO	13.8	20.5	19.9	20.5	12.8
LLL	6.6	14.7	13.2	12.9	9.1
OOL	8.3	9.6	8.8	8.4	7.6
LLP	8.7	9.7	10.4	11.4	8.3
PLO	10.6	9.1	9.3	11.1	11.1
LLLn	6.0	5.9	6.8	5.9	6.2
LnLO	7.0	5.5	5.8	7.6	7.2
LLS	3.8	3.3	4.0	2.0	3.8
LOS	3.7	3.0	2.9	3.4	4.2
PLnL	3.4	2.6	2.3	3.7	3.1
OOP	3.4	2.1	2.2	1.8	3.0
PPL	3.4	2.1	2.4	2.0	2.5
OOO	2.4	1.5	1.8	1.4	3.4
PLS	2.4	1.4	1.5	1.0	1.9
OOLn	2.1	1.3	1.3	0.8	2.6
PLnO	2.6	1.2	1.3	1.6	2.6
LnLS	1.3	0.9	0.8	0.0	1.5
PPO	1.8	1.0	0.9	0.7	1.2
LnLnL	1.2	0.8	0.8	0.7	1.4
OOS	1.4	0.7	0.8	0.8	1.3
POS	1.2	0.7	0.6	0.6	0.9
LnOS	0.9	0.4	0.4	0.0	0.9
LnLnO	0.8	0.4	0.4	0.3	1.0
SSL	0.5	0.2	0.3	0.4	0.4
PPLn	0.5	0.3	0.2	0.0	0.4
PLnS	0.4	0.2	0.1	0.0	0.3
LnLnP	0.3	0.2	0.1	0.0	0.3
PPS	0.3	0.2	0.1	0.3	0.2
PPP	0.3	0.2	0.1	0.1	0.2
SSO	0.3	0.1	0.1	0.1	0.2
LnLnS	0.1	0.1	0.0	0.0	0.1
SSP	0.1	0.1	0.0	0.1	0.1
LnLnLn	0.1	<0.05	<0.05	0.2	0.1
SSLn	0.1	<0.05	<0.05	0.0	0.1
SSS	<0.05	<0.05	<0.05	0.1	0.0
Other	0.4	0.3	0.2	0.10	0.0
Sum	100.0	100.0	100.0	100.0	100.0
Av. rel. err. % ($n = 20$)[c]	46.4	—	8.6	23.9	46.5

[a]Abbreviations and column headings as in Table 1.
[b]TG composition obtained by application of response factors calculated from the synthetic mixture. Response factors were calculated by dividing the TG amount calculated from a random distribution of the FA in the synthetic mixture by the TG amount determined by LC/MS.
[c]Average relative error for the twenty most abundant TG species.

TABLE 4
Fatty Acid Percentage Composition of Randomized Soybean Oil TG[a]

FA	From raw MS TG	From calibrated GC–FID	From GC–FID-adjusted TG	From LC–FID TG	TG position 2[b]
Po	0.2	0.1	0.1	0.0	0.0
P	15.4	11.6	11.9	12.5	13.8
Ln	9.8	7.0	7.3	7.4	4.5
L	42.0	52.7	52.2	52.3	49.5
O	26.8	24.6	24.5	24.6	27.7
S	5.8	3.9	4.1	3.2	4.5
Sum	100.0	100.0	100.0	100.0	100.0
Avg. relative error %	29.9	—	2.2	6.5	—

[a]Abbreviations as in Table 1. Column headings as in Table 2.
[b]FA composition at glycerol carbon 2 determined by GC–FID analysis of the products of lipase hydrolysis. Perfect agreement with the total FA composition determined by GC–FID indicates complete randomization.

sample by the raw APCI–MS percentage composition. The response factors thus obtained were applied to the raw APCI–MS data for the normal SBO. The resultant TG composition is given in Table 5. The TG composition agrees very well with the TG composition obtained using response factors calculated from the GC–FID composition determined for the normal SBO. The FA composition calculated from the randomized-SBO-normalized TG composition, shown in Table 6, exhibited an average relative error of 6.5% per FA. This compares favorably with the results obtained from the GC–FID-derived response factor normalized data.

Despite the close similarity in the results obtained using response factors derived from randomized SBO vs. GC–FID data, the methods used to obtain the response factors were very different. The randomized SBO-derived response factors were dependent only on the GC–FID composition and statistical distribution determined for the randomized SBO sample, with no dependence on the GC composition actually determined for the normal SBO. On the other hand, the GC–FID-derived response factors had no dependence on the randomized SBO sample or on any statistical calculation, but depended only on the GC–FID data for the normal SBO. That the two approaches resulted in very similar TG compositions, and yielded similar calculated FA compositions indicated that both quantitation methods may be used to produce results which provide less average relative error than LC–FID results.

Randomized lard. An LC/APCI–MS chromatogram of randomized lard is shown in Figure 9. The TG composition determined from this chromatogram is given in Table 7, as is the composition determined by LC–FID. Because this was a ran-

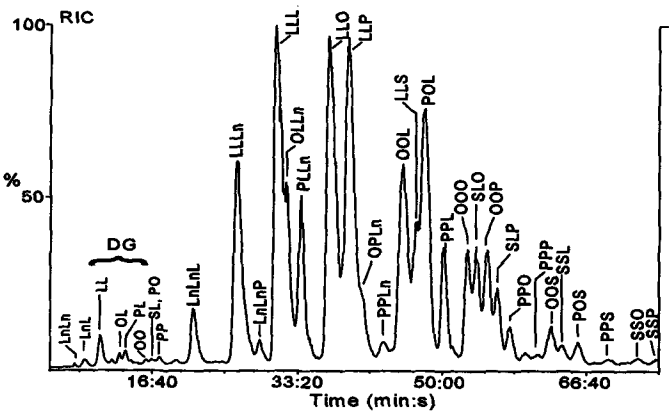


FIG. 8. Liquid chromatography/atmospheric pressure chemical ionization–mass spectrometry chromatogram of normal soybean oil. Peak labels identify DG or triglyceride molecular species. RIC, reconstructed ion chromatogram. See Figures 1, 2, and 3 for abbreviations.

TABLE 5
Percentage Composition of Normal Soybean Oil (SBO) TG[a]

TG	Raw MS %	GC–FID RF adjusted %	Randomized-SBO-adjusted %[b]	LC–FID %
LLO	11.2	17.7	17.3	16.4
LLL	6.1	13.6	14.0	15.3
LLP	9.7	11.8	11.2	14.0
OOL	7.6	8.5	9.0	8.3
PLO	9.2	7.9	8.1	10.1
LnLO	8.2	6.3	6.6	6.1
LLLn	6.1	6.7	6.2	6.5
LLS	4.0	4.9	3.6	3.3
PLnL	4.1	2.5	3.3	3.9
LOS	3.4	2.9	2.9	3.1
OOP	4.0	2.5	2.5	0.9
PPL	3.5	2.4	2.3	2.2
OOO	3.2	2.5	2.1	3.0
PLnO	3.5	1.5	1.7	0.9
PLS	2.3	1.6	1.5	1.2
OOLn	2.1	1.2	1.3	0.8
LnLnL	1.5	0.8	1.0	0.9
LnLS	1.4	0.8	0.9	0.0
OOS	1.6	1.0	0.8	0.8
PPO	1.3	0.6	0.8	0.5
POS	1.0	0.5	0.6	0.2
LnLnO	1.1	0.4	0.5	0.4
LnOS	1.0	0.4	0.5	0.0
SSL	0.5	0.4	0.3	0.3
PPLn	0.5	0.1	0.3	0.0
LnLnP	0.4	0.1	0.2	0.0
PLnS	0.4	0.1	0.2	0.0
LnLnS	0.2	0.1	0.1	0.0
SSO	0.3	0.1	0.1	0.0
LnLnLn	0.1	<0.05	0.1	0.1
SSLn	0.1	<0.05	<0.05	0.0
PoPL	0.1	<0.05	<0.05	0.0
PoPO	<0.05	<0.05	<0.05	0.0
PPP	<0.05	<0.05	<0.05	0.2
PPS	<0.05	<0.05	<0.05	0.1
Other	0.3	<0.05	<0.05	0.3
Sum	100.0	100.0	100.0	100.0

[a]Abbreviations and column headings as in Table 1.
[b]TG composition obtained by application of response factors calculated from randomized soybean oil. Response factors were calculated by dividing the expected TG composition (calculated based on random distribution of FA) by the TG composition determined by LC/MS.

TABLE 6
Fatty Acid Percentage Composition of Normal Soybean Oil (SBO) TG[a]

FA	From raw MS TG	From calibrated GC–FID	From GC–FID-adjusted TG	From LC–FID TG	From random.-SBO-adjusted TG[b]	TG position 2
Po	0.2	0.0	0.0	0.0	0.0	0.0
P	15.2	10.9	11.6	12.6	12.0	1.2
Ln	11.4	7.3	7.5	7.1	8.3	6.4
L	40.7	53.0	52.3	54.2	51.5	66.3
O	26.8	24.8	24.1	22.8	24.2	24.6
S	5.7	4.1	4.4	3.4	4.0	1.4
Sum	100.0	100.0	100.0	100.0	100.0	100.0
Avg. relative error %	19.8	—	4.5	9.2	6.5	—

[a]Abbreviations as in Table 1, column headings as in Table 4.
[b]FA composition calculated from the TG composition adjusted using randomized soybean oil-derived response factors.

domized sample, the theoretical composition could be calculated from the statistical distribution of FA. The FA composition determined by calibrated GC–FID used for calculation of the statistically expected TG composition is given in Table 8. The expected TG composition calculated from the FA composition is given in Table 7. Comparison of the FA composition at carbon 2 of the TG of the randomized lard to the total FA composition, Table 8, indicated that the lard was well randomized, in contrast to the FA composition at carbon 2 for the normal lard. The FA composition calculated from the "raw" TG composition determined by APCI–MS is given in Table 8. The FA composition determined by calibrated GC–FID was divided by the FA composition calculated from the "raw" TG composition to produce the following response factors for each of the FA: M = 1.66; Po = 1.00; P = 1.80; L = 1.90; O = 2.01; S = 1.73. Response factors for each of the TG molecular species was obtained by multiplying the appropriate FA response factors together, as described above. The TG composition resultant from application of the TG response factors derived from these FA response factors is given in Table 7. Because of the small amounts of linolenic acid and C_{20} fatty acids distributed throughout possible TG molecular species, these were not quantitated in the EIC for the lard samples, but several molecular species were identified by their fragments and pseudo-molecular ions. The average relative error for the 35 components which made up 99.6% of the quantitated composition was 10.1% per TG for the GC–FID-adjusted composition, compared to 31.4% per TG and 28.9% per TG for compositions determined by unadjusted APCI–MS data and LC–FID data, respectively. TG species which were not identified by LC–FID, and so had 100% relative error, were not included in the calculation of the average relative error for that detection method. Numerous species present at near 1% in composition were not identified in the LC–FID chromatograms because they eluted undetected under larger peaks. Many such species were conclusively identified from APCI–MS data, using EIC.

The FA compositions which were calculated from the TG compositions reported in Table 7 are shown in Table 8. As was the case for all samples discussed above, the average relative error for the FA composition calculated from the GC–FID-adjusted TG composition showed the least average relative error of the quantitation methods. The FA composition calculated from the LC–FID TG composition exhibited lower amounts of myristic and palmitoleic acids present than were determined by calibrated GC–FID. This is a result of the inability of the LC–FID detector to resolve the many minor components containing these FA which coeluted with components present in larger amounts. The EIC obtained from the APCI–MS data allowed ready identification, by $[DG]^+$ and $[M + 1]^+$ *m/z*, of numerous myristic and palmitoleic acid-containing species which coeluted with major species. This re-

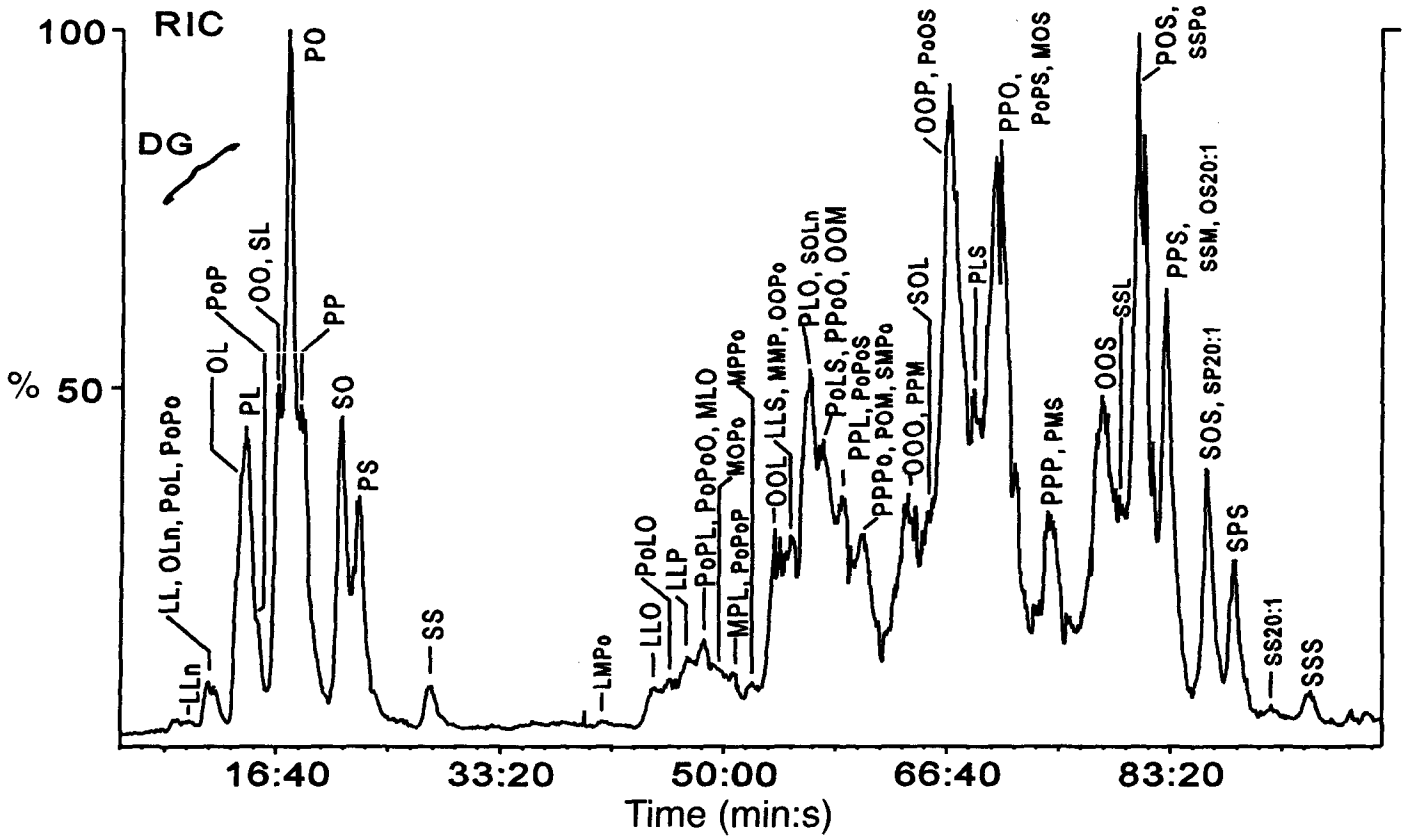


FIG. 9. Liquid chromatography/atmospheric pressure chemical ionization–mass spectrometry chromatogram of randomized lard. Peak labels identify individual DG and triglyceride molecular species. DG, formed during the randomization, eluted first. See Figures 1, 2, 3, and 8 for abbreviations.

sulted in excellent agreement between the response factor-adjusted APCI–MS data and the GC–FID data.

Normal lard. A chromatogram obtained by LC/APCI–MS of normal lard is shown in Figure 10. The TG composition determined from this chromatograms is given in Table 9. As in the case of normal SBO, two methods for calculation of response factors were used for quantitation of normal lard. Response factors were calculated by comparison of the FA composition determined by GC–FID, given in Table 10, to the FA composition calculated from the TG composition, and response factors were calculated from comparison of the theoretical composition of randomized lard to the composition determined by APCI–MS. The FA response factors calculated by dividing the FA composition calculated from the TG composition determined by APCI–MS by the FA composition determined by GC–FID, normalized to the smallest, were: M = 1.79; Po = 1.00; P = 1.84; L = 1.92; O = 1.91; S = 1.50. Response factors for each of the TG molecular species were obtained by multiplication of the appropriate FA response factors. The resultant response factor-adjusted TG compositions are given in Table 9. The adjusted APCI–MS compositions are in good agreement with each other, in contrast to the composition obtained using LC–FID. As with the randomized lard, numerous TG species present in amounts as high as 1.5% were not identified by LC–FID because they eluted undetected under larger chromatographic peaks. As with the randomized lard, this resulted in a FA composition calculated from the LC–FID TG composition which contained smaller amounts of myristic and palmitoleic acids than determined by GC–FID. The FA compositions calculation from the response-factor-adjusted APCI–MS data showed good agreement with the FA composition determined by calibrated GC–FID.

DISCUSSION

The analysis of the mono-acid TG presented above demonstrated that, for individual TG of interest, calibration curves having good correlation coefficients could be constructed. Even simple natural fats and oils, however, contain enough TG molecular species that it becomes impractical to construct calibration curves for all species of interest. Thus, alternative quantitation approaches were necessary which were targeted at more complicated samples. The trends observed in the slopes of the calibration curves, in correlation with the trends observed in the MS fragmentation patterns of TG with varying amounts of unsaturation, demonstrated that the quantitation of TG is distinctly related to the qualitative appearance of the spectra obtained using APCI–MS.

Quantitation of a complex sample generally depends on

TABLE 7
Percentage Composition of Randomized Lard TG[a]

TG	Raw MS % (n = 6)	Statistical % from GC–FID	GC–FID RF adjusted %	LC–FID %
OOP	12.3 ± 0.9	14.8	14.6	17.5
POS	11.2 ± 0.8	12.1	11.4	12.4
PPO	10.3 ± 0.9	10.9	10.9	12.5
OOS	7.4 ± 0.3	8.3	8.4	12.4
OOO	5.6 ± 1.2	6.7	7.3	7.5
PLO	4.7 ± 0.4	5.7	5.3	5.8
PPS	5.3 ± 0.7	4.5	4.8	3.9
OOL	3.7 ± 0.4	3.9	4.6	3.4
SSO	3.9 ± 0.4	3.4	3.8	2.9
LOS	2.5 ± 0.3	3.2	2.7	4.0
PPP	3.2 ± 0.3	2.7	3.0	1.4
SSP	2.8 ± 0.4	2.5	2.5	1.6
PLS	2.3 ± 0.3	2.4	2.2	3.4
PPL	2.0 ± 0.3	2.1	2.0	1.0
PoPO	3.2 ± 0.4	2.0	1.9	2.9
MPO	1.5 ± 0.3	1.4	1.5	0.0
OOPo	1.8 ± 0.2	1.3	1.2	0.0
PoOS	1.9 ± 0.3	1.1	1.1	0.0
OOM	0.8 ± 0.1	1.0	0.8	0.0
PoPS	1.8 ± 0.1	0.8	0.9	0.0
MOS	0.7 ± 0.1	0.8	0.7	0.0
LLO	1.0 ± 0.2	0.8	1.1	0.5
PPPo	1.4 ± 0.3	0.7	0.7	0.0
SSL	0.7 ± 0.1	0.7	0.7	0.0
MPS	0.7 ± 0.1	0.6	0.6	0.0
LLP	0.5 ± 0.1	0.6	0.6	0.5
PPM	0.6 ± 0.1	0.5	0.5	2.4
PoLO	0.8 ± 0.1	0.5	0.5	0.3
SSS	0.6 ± 0.1	0.5	0.5	0.8
PoPL	0.6 ± 0.0	0.4	0.3	0.0
MLO	0.3 ± 0.1	0.4	0.3	0.0
LLS	0.3 ± 0.0	0.3	0.3	1.6
MPL	0.3 ± 0.0	0.3	0.3	0.0
SSPo	0.7 ± 0.1	0.2	0.3	0.0
PoLS	0.3 ± 0.1	0.2	0.2	0.0
Other	0.4	1.9	1.4	1.2
Sum	100.0	100.0	100.0	100.0
Av. rel. err. % (n = 35)	31.4	—	10.1	28.9

[a]Abbreviations and column headings as in Table 1. Other abbreviations: M, myristic acid (14:0); Po, palmitoleic acid (16:1).

TABLE 8
Fatty Acid Percentage Composition of Randomized Lard TG[a]

FA	From raw MS TG	From calibrated GC–FID	From GC–FID-adjusted TG	From LC–FID TG	TG position 2
M	2.2	2.0	1.9	0.8	1.0
Po	4.9	2.7	2.7	1.1	2.5
P	30.4	29.9	29.8	29.7	29.5
Ln	0.0	0.3	0.0	0.0	0.3
L	7.6	7.9	8.0	7.8	8.9
O	37.1	40.6	40.9	44.0	40.7
S	17.7	16.7	16.6	16.6	17.1
Sum	100.0	100.0	100.0	100.0	100.0
Avg. relative error %	19.0	—	1.0	21.7	

[a]Abbreviations as in Table 1 and column headings as in Table 4. Other abbreviations as in Table 7.

analysis of samples of known composition containing all species of interest. It was hoped that a complex synthetic mixture could be produced which, by nature of the method of its synthesis, would have a known composition, which could then be used as a calibration mixture. Toward this end, the mixture of 35 TG derived from five FA was produced. However, as the results in Table 3 showed, it quickly became apparent that there was sufficient concentration dependence in the response of the TG that such a universal calibration mixture was not adequate. Examination of the calibration curves confirmed that there was a noticeable change in slope of the points at lower concentration vs. those at higher concentration, which resulted in a concave appearance of the curves. Thus, while overall the calibration curves indicated linear responses over a broad concentration region, within different regions of the curves enough difference in slope was present to indicate that there was potential for problematic effects in calculation of percentage composition in mixtures containing TG present in disparate percentages.

Another method for production of a complex mixture of known composition was necessitated. Just as the composition of the synthetic mixture was given by the statistical distribution of the FA used for the synthesis, the composition of a completely randomized sample is given by the statistical distribution of its FA composition. Thus, the FA composition determined by calibrated GC–FID could be used to determine the composition of a completely randomized sample. This approach has the advantage that, if the randomized sample was used for calibration of a similar nonrandomized sample, the FA composition should be the same, so that the concentration differences between TG species arising from those FA in the two samples would not be great. However, unlike the synthetic mixture, for which there was no theoretical reason to believe that a random distribution was not achieved, a randomized fat or oil sample depends on dissociation and then reassociation of FA from/to the glycerol backbone, so independent steps had be taken to confirm that randomization occurred. Lipase hydrolysis has become a commonly used tool for determination of the positional distribution of FA within TG species, by differentiating between FA at the carbon 1,3 positions vs. the carbon 2 position. While not perfect, this approach is generally considered useful to indicate the degree of randomization achieved. For a completely randomized sample, the composition determined for the FA at carbon 2 equals the total FA composition. This is in distinct contrast to most natural fats and oils, which show distinct preferential placement of FA, depending on the type of fat or oil. The differences in FA placement in normal vs. randomized oils is seen by comparison of the lipase data for randomized vs. normal SBO in Tables 4 and 6, respectively. Normal SBO showed much less palmitic acid in the carbon 2 position vs. the total FA composition, but more linoleic acid in this position vs. the total FA composition. In contrast, the randomized sample showed much better agreement between the total FA

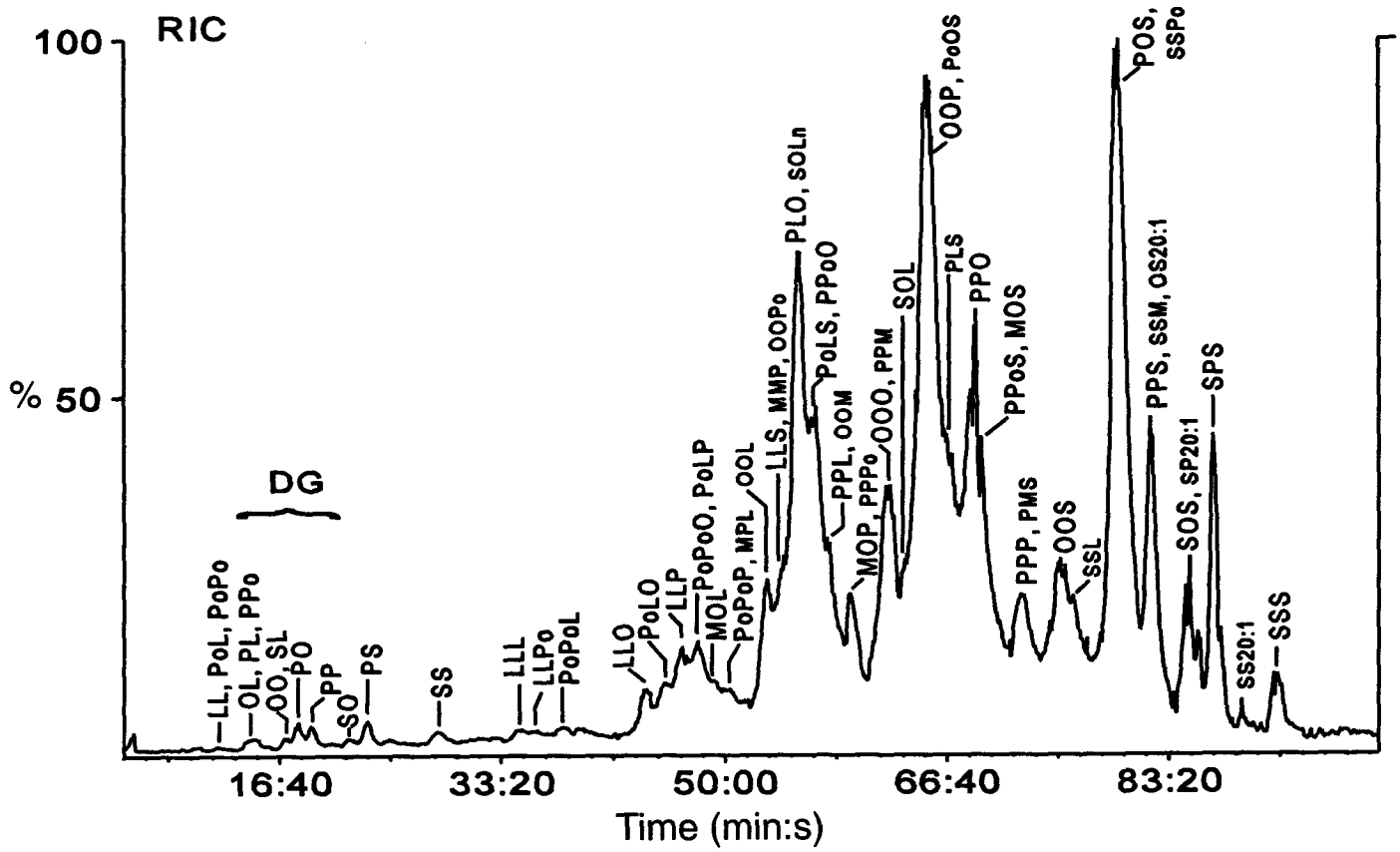


FIG. 10. Liquid chromatography/atmospheric pressure chemical ionization–mass spectrometry chromatogram of normal lard. Peak labels identify DG or triglyceride molecular species. See Figures 1, 2, 3, and 8 for abbreviations.

composition and the composition at carbon 2, though the agreement was not absolute, indicating that the randomization was not absolute. Opposite trends were observed in the lipase data for randomized vs. normal lard. Palmitic acid was preferentially incorporated into the 2-position of normal lard vs. randomized lard, shown in Tables 8 and 10, respectively, while less of oleic and linoleic acids appeared in the 2-position. More importantly, there was excellent agreement between the FA composition at the 2-position vs. the total FA composition, indicating a high degree of randomization.

The lipase data indicated that calculation of the TG composition of the randomized samples from the statistical distribution of the FA composition was expected to provide a good representation of the TG composition of the sample. Then, using the calculated TG composition to calculate response factors for each TG resulted in an adjusted TG composition equal to the random distribution. When the response factors thus derived were applied to the nonrandomized samples, good agreement was obtained between this method and another quantitation method, which used response factors based on FA. Importantly, the FA composition of the normal samples calculated from the TG composition obtained by application of the response factors calculated from the randomized samples showed excellent agreement with the FA composition obtained by calibrated GC–FID of the normal samples. The agreement with the GC–FID FA composition was better than that obtained from the TG composition determined by LC–FID, which was also in good agreement with the GC–FID results. However, the LC–FID detector did fail to identify numerous TG components. In the lard samples, most of these unidentified species contained myristic and palmitoleic acids, so these FA produced large relative error in the FA composition.

The response factors calculated using the synthetic mixture and the randomized samples all reflected the dependence of the qualitative behavior of the response factors on the fragmentation behavior of the TG, in combination with practical complications such as isotope peaks chromatographically coeluted with certain FA. The FA compositions calculated from the TG composition determined by APCI–MS also showed the same trends. Thus it seemed reasonable to use the FA composition for calculation of response factors to compensate for overrepresented and underrepresented FA. Several approaches were tried, but the best results came from simple multiplication of individual FA response factors together to produce TG response factors. The results obtained for all samples using this simple approach were in good agreement with calculated results, and the FA compositions calculated from the adjusted TG compositions were also in good agreement with the GC–FID results. Like the approach

TABLE 9
Percentage Composition of Normal Lard TG[a]

TG	Average raw MS % ($n = 6$)	GC–FID RF adjusted %	Randomized-lard-adjusted %	LC–FID %
OOP	17.1 ± 2.0	20.9	20.4	25.7
POS	16.7 ± 0.4	16.1	18.1	18.2
PLO	5.6 ± 0.5	6.9	6.8	7.5
PPO	6.2 ± 0.8	7.3	6.5	7.6
OOS	4.6 ± 0.5	4.6	5.1	7.2
OOO	3.8 ± 0.6	4.9	4.6	5.2
SSP	4.4 ± 0.4	3.4	3.9	2.9
PPS	4.5 ± 0.6	4.2	3.8	3.5
PLS	3.1 ± 0.3	3.0	3.2	5.0
OOL	2.7 ± 0.3	3.4	2.8	2.3
LOS	1.8 ± 0.1	1.8	2.3	1.0
SSO	2.5 ± 0.1	1.9	2.2	1.5
OOPo	2.5 ± 0.5	1.6	1.8	0.0
PoPO	2.8 ± 0.4	1.8	1.7	3.6
PPP	1.9 ± 0.3	2.2	1.6	1.2
PoOS	2.6 ± 0.2	1.4	1.5	0.0
OOM	1.0 ± 0.1	1.2	1.3	0.0
PPL	1.1 ± 0.2	1.4	1.2	1.0
MOS	0.9 ± 0.3	0.9	1.1	0.0
MPO	1.1 ± 0.2	1.3	1.0	0.0
SSS	1.2 ± 0.2	0.7	0.9	0.9
PoPS	1.5 ± 0.2	0.8	0.7	0.0
PoLO	1.1 ± 0.1	0.7	0.7	0.4
LLP	0.6 ± 0.1	0.8	0.6	0.8
LLO	0.8 ± 0.1	1.0	0.6	0.5
MPS	0.7 ± 0.2	0.6	0.6	0.0
MLO	0.4 ± 0.1	0.5	0.6	0.0
SSL	0.5 ± 0.1	0.4	0.4	0.0
PoPL	0.5 ± 0.0	0.3	0.3	0.0
PoLS	0.5 ± 0.1	0.2	0.3	0.0
PPM	0.3 ± 0.0	0.4	0.3	1.5
PPPo	0.6 ± 0.1	0.3	0.3	0.0
LLS	0.2 ± 0.0	0.2	0.3	1.5
MMO	0.4 ± 0.7	0.4	0.3	0.0
SSPo	0.7 ± 0.1	0.3	0.2	0.0
Other	3.0	2.1	1.9	1.0
Sum	100.0	100.0	100.0	100.0

[a]Abbreviations as in Table 1 and column heading as in Table 5. Other abbreviations as in Table 7.

TABLE 10
Fatty Acid Percentage Composition of Normal Lard TG[a]

FA	From raw MS TG	From calibrated GC–FID	From GC–FID-adjusted TG	From LC–FID TG	From random. SBO adjusted TG[b]	TG position 2
M	2.4	2.1	2.4	0.5	2.2	2.3
Po	5.1	2.8	2.9	1.4	2.8	3.9
P	28.8	30.3	30.1	31.8	29.0	57.3
Ln	<0.05	0.3	0.0	0.0	0.1	0.4
L	7.4	7.9	8.2	7.7	7.7	5.1
O	36.9	40.1	40.1	42.6	40.2	22.9
S	19.3	16.4	16.3	16.1	18.0	8.1
Sum	100.0	100.0	100.0	100.0	100.0	100.0
Avg. relative error %	22.2	—	3.9	24.0	3.8	

[a]Abbreviations as in Table 1 and column headings as in Table 4. Other abbreviations as in Table 7.

which used response factors calculated from randomized samples, this approach gave composition information for many species which were undetected by LC–FID. This approach had an advantage over the use of response factors derived from the randomized samples: it was equally applicable to randomized and to normal samples alike. The response factors were calculated based on the net over- or under-response of the FA, with no dependence on statistical distribution. Thus, whether a sample has been completely randomized or not was irrelevant, because the FA composition was determined using GC–FID. Response factors produced from the randomized mixtures were dependent on the statistical distribution of the FA, so lipase hydrolysis, or other method for confirmation of the randomization, was necessary.

Two methods for quantitation of TG composition using LC/APCI–MS data have been presented which provided good agreement to each other and to expected results. They both yielded FA compositions which were in good agreement with FA compositions obtained using GC–FID analysis. The choice of which method is most appropriate for a particular use depends on the goals of the total analytical scheme being employed. Simple analyses of fat and oil TG composition may make use of the simplicity of calculation of response factors with only GC–FID data required. On the other hand, some analyses, such as studies of genetically modified seed oils or dietary studies, often require the use of additional techniques, like lipase hydrolysis, as routine aspects of the analytical approach, so this information should be used, if available. Both of the methods described yielded results which showed less relative error than the commonly used quantitation method of LC–FID analysis. Although the FA compositions calculated from the TG compositions obtained using LC–FID were in good agreement with the FA compositions obtained by GC–FID (except for FA present at moderately low levels), the TG composition obtained by LC–FID contained numerous omissions of TG species which were conclusively identified using LC/APCI–MS. The two-dimensional nature of the FID, as well as ELSD and other detectors, imposes inherent limitations on their ability to fully characterize complex fat and oil samples. A simple and versatile analytical method like LC/APCI–MS makes mass spectrometric detection an attractive alternative for complete qualitative and quantitative characterization of complex TG mixtures.

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Postparturition Changes in the Triacylglycerols of Cow Colostrum

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ABSTRACT: The changes in the triacylglycerol (TAG) composition of colostrum fat of three cows were studied. In addition to the determination of fatty acid composition by gas chromatography, the distribution of TAG according to the acyl carbon number (ACN) and molecular weight was analyzed utilizing both supercritical fluid chromatography (SFC) and ammonia negative-ion chemical ionization mass spectrometry (MS). Colostrum TAG contained substantially less stearic and oleic acids and more myristic and palmitic acids than the normal Finnish milk fat. The major trends in the changes of fatty acids and TAG were similar for each cow, although clear differences between individuals were found. During the first week of parturition, the proportions of short-chain fatty acids (C_4–C_{10}) typically increased as well as those of stearic and oleic acids, whereas the relative amounts of C_{12}–C_{16} acids decreased, especially those of myristic and palmitic acids. Distinct changes occurred also in TAG distributions: the proportions of molecules with ACN 38–40 increased and those with ACN 44–48 decreased. Although there were distinct differences between individuals shortly after delivery, both the fatty acid compositions and TAG distributions of the milk samples of the cows started to resemble each other after one week. The theoretical profiles of colostrum TAG calculated based on the fatty acid compositions differed clearly from the ACN distributions analyzed by SFC and MS. Thus, the analysis of TAG is essential, because the changes in molecular species composition of colostrum TAG cannot be estimated according to the fatty acid analysis alone. *Lipids 31*, 937–943 (1996).

The composition of milk fat triacylglycerols (TAG) has been extensively studied. However, most often information on the fatty acid composition, produced by gas chromatography (GC), has been found to be adequate. More specific information about milk fat has been achieved by analyzing the intact TAG by chromatographic methods, such as GC on both nonpolar (1,2) and polar stationary phases (3–5), and by high-performance liquid chromatography (HPLC) on reversed-phase columns (2,6–9) and ion-exchange columns loaded with silver ions (7,8,10–12). Furthermore, supercritical fluid chromatography (SFC) has been proved to be a useful technique for the analysis of TAG of various samples, including milk fat (13). In addition, milk fat TAG have been analyzed utilizing mass spectrometry (MS) in different modes usually in combination with chromatographic separation techniques (4,6,8,9,14–16). Chromatography prior to MS improves the interpretation of the results of complex mixtures. For some purposes, ammonia negative-ion chemical ionization MS, without chromatographic separation, has been found to be a rapid and sensitive technique for the analysis of TAG (17,18).

The colostrum of various species differs clearly from the mature milk. Some studies have been focused on the determination of fatty acid composition in cow colostrum and changes in the proportions of fatty acids after parturition (19–23). However, the TAG composition of colostrum has not been studied extensively. The present study was designed to provide information on the changes occurring in the composition of cow colostrum during the first week after parturition. The fatty acid compositions were determined by GC, and the TAG were separated according to the acyl carbon numbers (ACN) by SFC on a nonpolar column. In addition, molecular weight species of TAG were analyzed by ammonia negative-ion chemical ionization MS. The theoretical ACN distribution of TAG was calculated based on the fatty acid compositions and compared with that obtained on SFC and MS.

EXPERIMENTAL PROCEDURES

Materials. Milk samples were received from three cows, two of which were Ayrshires (cow 1, cow 2) and one Frisian (cow 3). Cow 2 was a descendant of cow 1. The cows were bred in Finland on the same farm and kept on the same diet consisting of oat meal, silage, concentrate, dry hay (*ad libitum*), minerals, and vitamins. The cows calved during a 10-d period in February 1993. Cow 1 had its fourth parturition, whereas cow 2 had its first and cow 3 its fifth calving. The milk samples were collected after every 12 h during the first week after parturition and stored at −20°C until the fat was separated by centrifugation and phase-exchange using sodium sulphate. TAG were purified from the total lipids by elution from short columns of Florisil™ with 10 mL of *n*-hexane/diethyl ether (4:1, vol/vol). Purified TAG fractions of 1 mg were dissolved in 1 mL of *n*-hexane for mass spec-

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Abbreviations: ACN, acyl carbon number; FID, flame-ionization detector; GC, gas chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; SFC, supercritical fluid chromatography; TAG, triacylglycerols.

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trometric analyses and for preparation of fatty acid methyl esters. An aliquot of the total lipid fraction was diluted in dichloromethane for supercritical fluid chromatographic analyses. All solvents were of HPLC-grade and were supplied by Merck (Darmstadt, Germany) or Rathburn (Walkerburn, Scotland).

GC. Fatty acid methyl esters of the TAG were prepared by sodium methoxide-catalyzed transesterification as described elsewhere (7). The analyses were obtained on a Hewlett Packard 5890 gas chromatograph (Palo Alto, CA), equipped with a split injector (split ratio 35:1) and a flame-ionization detector (FID). An OV-351 column (25 m × 0.20 mm i.d. with 0.20 μm film; HNU-Nordion Instruments Ltd., Helsinki, Finland) was used for GC with a three-step temperature programming initialized at 35°C with a 3-min isothermal period followed by a linear temperature increase at 10°C min^{-1} from 35 to 160°C, 2°C min^{-1} from 160 to 190°C, and 5°C min^{-1} from 190 to 230°C. The injector and detector temperatures were 225 and 240°C, respectively. Helium was used as a carrier gas with a column flow rate of 30 cm s^{-1} measured with propane at the initial conditions of the chromatographic program. Fatty acids were identified by reference to three standard mixtures of fatty acid methyl esters (ME61, ME68, and BR1; Larodan Fine Chemicals AB, Malmö, Sweden). The response correction factor for each fatty acid methyl ester, used for the conversion of percentage peak area to mol%, was determined by analyzing the reference mixture ME61.

SFC. The SFC analyses were conducted with a Lee Scientific Series 600 supercritical fluid chromatograph (Dionex, Salt Lake City, UT) equipped with an FID. The temperature of the FID was held at 340°C, and nitrogen was used as a make-up gas. An electrically and pneumatically controlled Valco switching valve (Valco Instruments Co. Inc., Houston, TX) with an internal loop volume of 1.0 μL was used for timed split/dynamic split injections with a loop-open time of 0.6 s. Frit restrictors (30 cm × 50 μm i.d.; Dionex) were installed both at the dynamic split outlet and at the detector end of the analytical column. SFC-grade CO_2 (Scott Specialty Gases, Plumsteadville, PA) was used as a carrier fluid with a column flow rate of 0.37 mL min^{-1} measured with propane at the initial conditions of the chromatographic program. An SB-Octyl-50 column (10 m × 50 μm i.d. with 0.25 μm film; Dionex) was used with linear density programming of CO_2 from 0.140 g mL^{-1} with a rate of 0.010 g mL^{-1} min^{-1} at a constant temperature of 140°C for the elution of TAG. Samples of 40 mg were dissolved in 1 mL of dichloromethane. The ACN distributions of TAG in the figures were means of three determinations without applying response factors.

MS. Ammonia negative-ion chemical ionization of TAG was obtained on a Finnigan MAT (San Jose, CA) TSQ-700 triple quadrupole instrument with a direct exposure probe for sample introduction (17,18). An aliquot of 1.0 μL of TAG dissolved in *n*-hexane was applied to the rhenium wire of the direct exposure probe. The probe was introduced into the ion source after the sample solvent was evaporated at ambient temperature. The sample was vaporized in the ion source by heating the rhenium wire with the current rate of 40 mA s^{-1}. The pressure of ammonia (≥ 99.998%; Prax Air, Oevel, Belgium) was 8500 mtorr, and the ion source temperature was 200°C. The electron energy applied was 70 eV and the filament current 400 μA. The *m/z* values were scanned from 450 to 950 with a scanning time of 0.5 s. Ammonia negative-ion chemical ionization MS of TAG yielded simple spectra containing abundant $[M - H]^-$ ions only in the scanned molecular weight region. Scans containing ions representing deprotonated TAG were averaged in each analysis. The abundance of the $[M - H]^-$ ions in the averaged spectrum was corrected according to the ^{13}C and ^{18}O isotopes, before expressing the results as the mean of four analyses. No corrections were made according to the mass spectrometric response factors of TAG.

RESULTS AND DISCUSSION

Fatty acid compositions. The changes in the fatty acid composition of colostrum TAG during the first week after parturition were determined by GC as fatty acid methyl esters. Altogether 26 fatty acids were separated and identified from the colostrum fat samples. The most abundant fatty acids were butyric acid, myristic acid, palmitic acid, stearic acid, and oleic acid, together representing more than 75 mol% of the total fatty acids in each sample. Compared with the composition of normal Finnish milk fat (8), the colostrum TAG were substantially lower in stearic and oleic acids, and richer in myristic and palmitic acids.

The major trends in the changes of the proportions of fatty acids were similar for each cow, although variation between individuals was found (Fig. 1). The proportions of the short-chain fatty acids, from C_4 to C_{10}, increased after parturition with the exception of cow 1. The relative amounts of fatty acids with 12–16 acyl carbon atoms typically decreased, especially those of myristic acid and palmitic acid. Furthermore, the proportions of stearic acid, oleic acid, and *cis*-vaccenic acid (data not shown) increased after parturition with the exception of cow 2. The relative amounts of linoleic acid and α-linolenic acid (data not shown) were less than 2 and 0.6 mol%, respectively, in each sample, and only minor changes in their proportions were observed. In general, the proportions of different fatty acids started to resemble each other more and approached the composition of normal Finnish milk fat after one week, even though there were great differences between individuals shortly after delivery. For example, the proportion of $C_{18:1}$ fatty acids in the milk samples of the three cows approached the value of 20 mol% which is typical for Finnish winter milk.

Our results are in good accordance with those reported by Senft and Klobasa (20) and Baše *et al.* (22). There are also contradictory results as reviewed by Christie (24) and Hawke and Taylor (25): the proportion of palmitic acid has been reported to remain relatively constant or increase slightly, and the relative amounts of stearic acid and oleic acid have been found to diminish after calving. During early lactation, the fatty acids are mobilized extensively from the adipose tissue

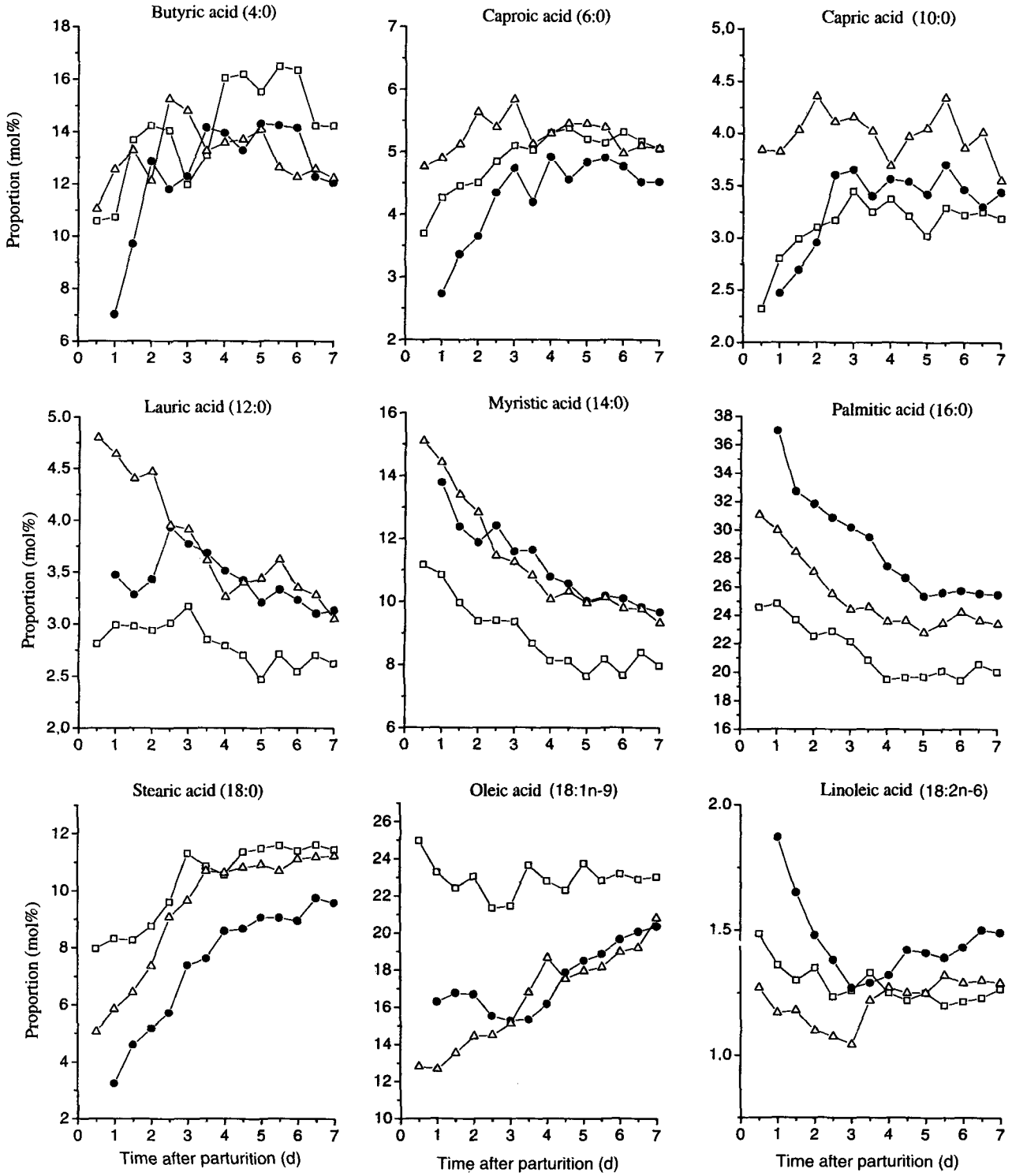


FIG. 1. Changes in the proportions (mol%) of fatty acids in the milk fat triacylglycerols of three Finnish cows (△ = cow 1, □ = cow 2, ● = cow 3) during the first week after parturition. The most abundant fatty acids of groups representing C_4–C_{10} acids, C_{12}–C_{16} acids, and C_{17}–C_{18} fatty acids are presented. The method for the analysis of fatty acids is described in the Experimental Procedures section.

for milk fat synthesis. A decline in the proportions of C_{18} fatty acids has been assumed to reflect the drop in the extent of lipid mobilization from the adipose tissue after parturition (24). The most abundant fatty acids in the tallow of Finnish cows are palmitic, stearic, and oleic acids. Thus, the diminished mobilization of storage lipids would partially explain the decrease in the proportion of palmitic acid, but not the observed increase in the proportions of stearic acid and oleic acid in the present study. The high content of myristic acid in the milk TAG shortly after delivery and the increase in the proportions

of short-chain fatty acids indicate distinct changes in the *de novo* synthesis of fatty acids after parturition.

Although significant changes in the proportions of individual fatty acids occurred during the first week after parturition, the proportions of total saturates (approximately 70 mol%) and unsaturates (approximately 30 mol%) were nearly constant. In general, the most distinct changes in fatty acids occurred during the first four days, after which the composition slowly approached that of normal milk fat. The fatty acid compositions, however, did not provide information concerning changes at the molecular level, thus, other techniques were required.

TAG distributions. The TAG compositions of the colostrum samples were measured with both SFC and MS. SFC separated TAG mainly according to the combined number of acyl carbons on a nonpolar stationary phase (Fig. 2). Colostrum TAG were separated into groups representing molecules with 26–54 acyl carbons. The presence of isobaric TAG, differing substantially in the chainlength of the fatty acyl residues, resulted in a partial separation within most of the ACN-groups. For example, 1,3-dipalmitoyl-2-butanoyl-*sn*-glycerol eluted well-separated after trilauroylglycerol as tested with reference compounds. In addition to the molecular association of fatty acids, the steric interactions between the fatty acid moieties and the stationary phase have an effect on the retention. The differences in unsaturation of the analytes were less important on the separation, causing only slight broadening of the chromatographic peaks. Figure 2 shows that the distribution of TAG changed clearly after parturition. For example, a decline in the proportions of molecules having from 42 to 48 acyl carbons was found. The repeatability of the analysis was acceptable, the coefficient of variation typically being less than 3% for the components corresponding to more than 1% of the total.

The advantage of MS was the unit resolution that made it possible to achieve information on the molecular species composition of TAG. The *m/z* values of the $[M - H]^-$ ions characterized the combined number of acyl carbons and double bonds in the acyl chains of TAG, and the abundances of the $[M - H]^-$ ions defined the proportions of different molecular weight species of TAG. In addition to the ACN distribution, the analysis of colostrum TAG by ammonia negative-ion chemical ionization MS produced information on the saturated, monoenoic, dienoic, and polyenoic TAG consisting of fatty acids with even or odd numbers of carbon atoms without chromatographic separation. TAG containing *cis*- or *trans*-fatty acids could not be separated with the method used.

Information on the ACN distribution of TAG was achieved from both SFC and MS determinations. Figure 3 shows as an example the measured ACN distributions of the TAG of the milk samples of cow 1. The proportions of different ACN-groups, analyzed either by SFC or MS, differed to some extent, which was due to the differences in molecular discrimination of the analytical techniques. Nevertheless, both methods provided similar trends for the changes of colostrum TAG, and therefore, are useful for biological screening purposes.

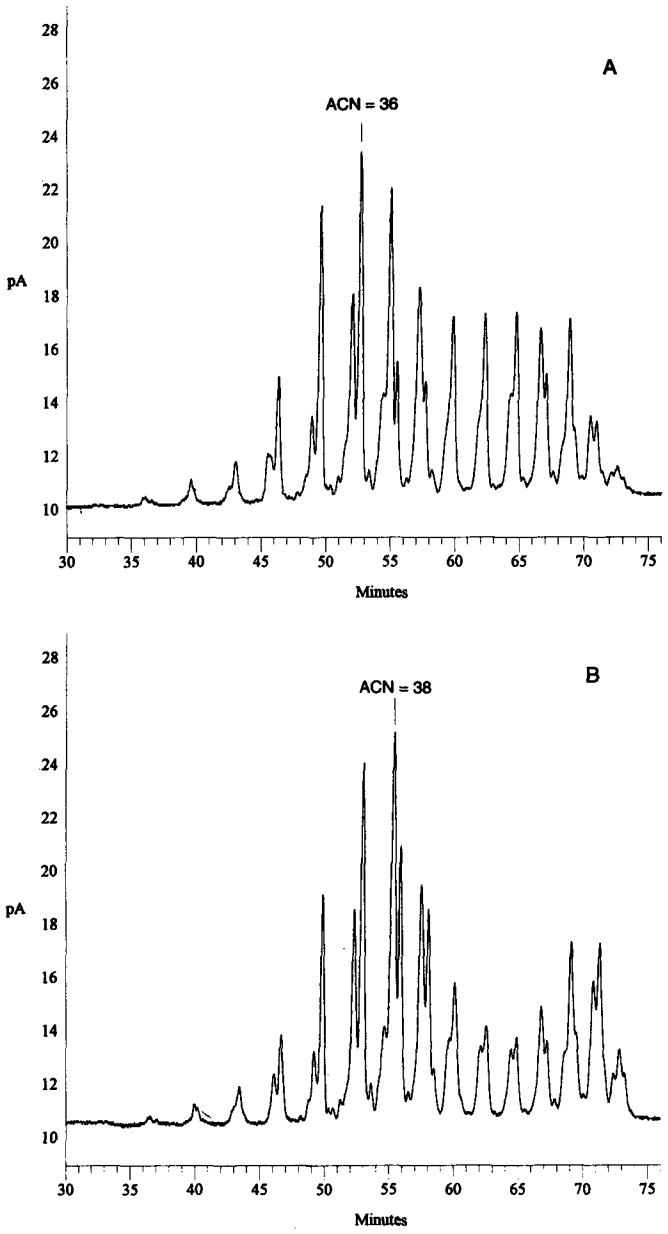


FIG. 2. Supercritical fluid chromatographic separation of the triacylglycerols of the colostrum samples: A, 1 d after parturition; and B, 7 d after parturition of the cow 1 on an SB-Octyl-50 column (10 m × 50 μm i.d. with 0.25 μm film; Dionex, Salt Lake City, UT). ACN, acyl carbon number.

The differences in the TAG profiles between the cows studied were marked; however, similarities were also found. The general distribution profile of TAG of each sample was bimodal. TAG with ACN 36–40 were the most abundant components among the molecules, which may contain butyric acid. The maximum of the TAG, which do not contain butyric acid, was in the region of ACN 46–52. The composition changed substantially during the first week after parturition: the proportions of TAG with ACN 38–40 increased and those with ACN 44–48 decreased. The alterations in the proportions of molecules with 50–54 acyl carbons varied from cow

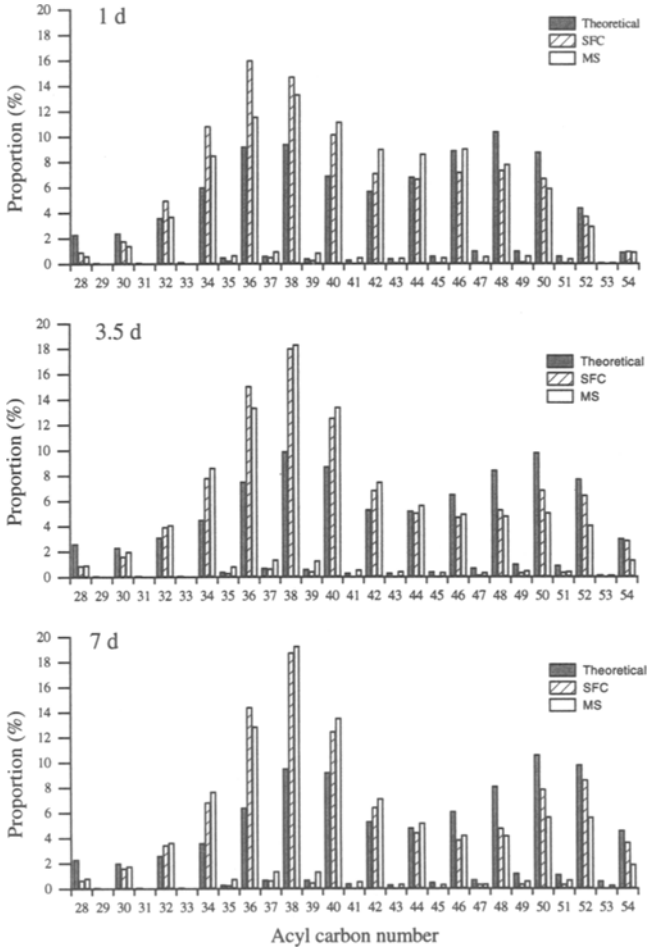


FIG. 3. Acyl carbon number (ACN) distribution of milk fat triacylglycerols 1, 3.5, and 7 d after parturition of the cow 1 determined by supercritical fluid chromatography (SFC) and mass spectrometry (MS). The theoretical ACN distribution of triacylglycerols is calculated according to the fatty acid compositions, assuming a 1,2,3-random distribution of fatty acids.

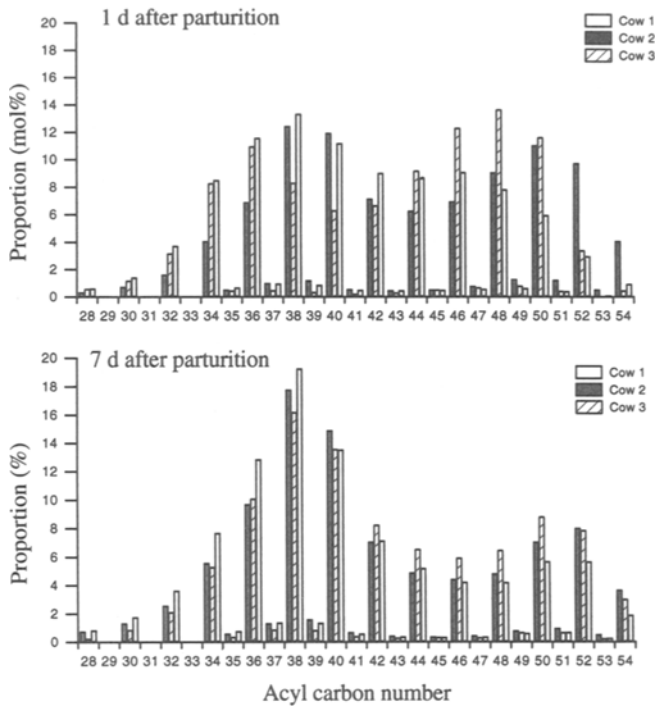


FIG. 4. Acyl carbon number distribution of milk fat triacylglycerols of three Finnish cows 1 and 7 d after parturition. The results are based on the mass spectrometric data.

to cow and may be explained by the great differences in the proportions of $C_{18:1}$ fatty acids between individuals (Fig. 1). Figure 4 presents the TAG distributions, based on MS-data, of the colostrum of each cow collected 1 d and 7 d after parturition. The TAG profiles showed distinct differences between individual cows shortly after delivery. After one week, the changes between the cows were clearly diminished and the compositions of milk TAG were much alike.

For comparison, the distribution of the TAG of each colostrum sample was calculated according to the fatty acid composition, assuming a 1,2,3-random distribution of fatty acids. The theoretical distributions of TAG clearly differed from those measured by SFC and MS (Fig. 3): the proportions of TAG with ACN 34–42 were underestimated and the proportions of TAG with ACN 28 and 46–52 overestimated. The nonrandom structure of TAG of mature milk is well known (26,27), and similar deviations from the random distribution have been reported elsewhere (9,28). The molecular association of fatty acids during the biosynthesis of milk fat TAG is not a random process; therefore, it is not possible to estimate the changes occurring in molecular species composition of TAG based on the fatty acid analysis alone.

In addition to ACN distributions obtained on SFC and MS, the mass spectrometric analysis provided information on differently unsaturated molecular species of TAG. The proportions of saturated, monoenoic, dienoic and trienoic TAG in the colostrum of the three cows studied are presented in Table 1. The differences in the TAG composition of the milk samples after 1 d of parturition of each cow were distinct. After one week of parturition, the TAG compositions were more alike; 32–44 mol% saturated TAG, 41–45 mol% monoenes, 12–19 mol% dienes, and 2–4 mol% trienes. This kind of composition of the TAG is typical for Finnish winter butter fat (8).

As an example, the changes in the distribution of saturated and monoenoic TAG in the milk samples of cow 1 are shown in Figure 5. The most abundant saturated components were 34:0, 36:0, and 38:0. The proportion of 38:0 increased after parturition in the colostrum of each cow, whereas the proportions of 44:0–48:0 declined. The changes in the relative amounts of 28:0–36:0 varied from cow to cow. The profiles of monoenoic TAG were bimodal, closely resembling the ACN profiles. The most abundant monoenoic TAG were 36:1, 38:1, 40:1, 46:1, 48:1, and 50:1. Typically, the proportions of TAG 28:1–40:1 increased and those of TAG 44:1–50:1 decreased after parturition.

The data presented in this study, i.e., changes in the fatty acid compositions and TAG profiles, improves our under-

TABLE 1
Changes in the Proportion (mol%) of Triacylglycerols, Differing in the Degree of Unsaturation, in the Milk Samples of Three Finnish Cows During the First Week After Parturition[a]

Triacylglycerols	Time after parturition (d)	Proportion (mol%) Cow 1	Cow 2	Cow 3
Saturated	1	51.6	27.4	41.6
	3.5	48.7	32.4	37.6
	7	44.0	32.4	36.5
Monoenes	1	37.2	45.2	43.8
	3.5	39.1	44.4	44.1
	7	41.1	44.7	43.2
Dienes	1	9.4	21.7	12.1
	3.5	10.3	18.7	15.0
	7	12.4	18.7	16.4
Trienes	1	1.7	4.8	2.3
	3.5	1.8	4.0	3.0
	7	2.3	3.8	3.3
Others	1	0.2	0.9	0.2
	3.5	0.1	0.5	0.3
	7	0.3	0.4	0.6

[a]Results calculated from mass spectrometric data.

standing of biological changes in colostrum TAG. Apparent differences between individuals were found, which may be due to genetic factors including the breed of a cow, the age, and the time of delivery. Changes in the fatty acid composition of the milk samples determined by capillary GC did not provide adequate information about the changes in TAG compositions. Comparisons of the ACN distributions of colostrum TAG, measured by SFC and MS, with the theoretical ones, based on the fatty acid compositions, clearly showed that the molecular association of fatty acids during the biosynthesis of milk fat is not a random process. Both SFC and MS are convenient techniques for the analysis of TAG. The ACN distribution of TAG is achieved directly from the SFC data sheet, whereas MS data providing more detailed information require extensive calculations. Therefore, sophisticated computer programs for the handling of MS data would be advantageous in routine analysis. The present study clearly showed that it was not possible to estimate the changes in the molecular species composition of colostrum TAG according to the fatty acid analysis alone. Instead, both SFC and MS analyses provided valuable information about TAG compositions in the colostrum.

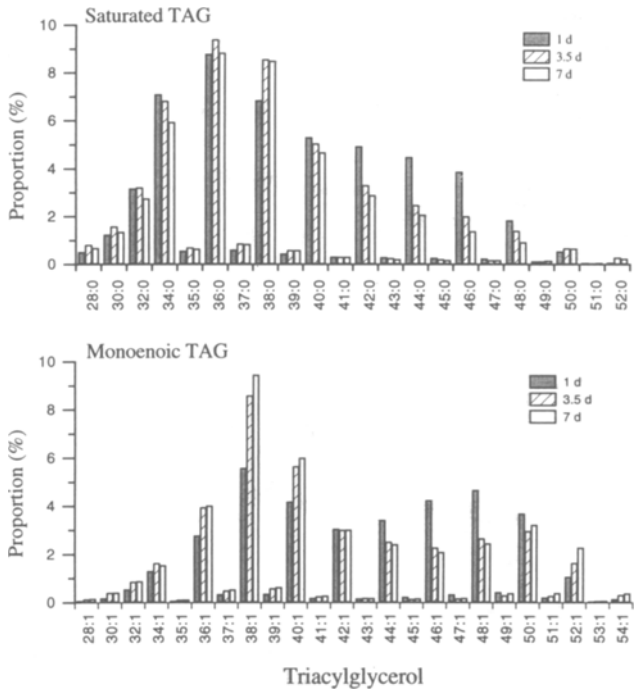


FIG. 5. Changes in the distribution of saturated and monoenoic triacylglycerols (TAG) in the milk fat of cow 1 during the first week after parturition (1, 3.5, and 7 d).

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Effect of Castration and Hormonal Supplementation on Cholesterol Cholelithiasis in the Male Hamster

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ABSTRACT: This study examined the effect of castration and dietary hormonal supplementation on cholesterol cholelithiasis in male hamsters. Animals fed a standard lithogenic diet developed cholesterol gallstones (17%) after 6 wk, while castrated hamsters did not form any stones. Addition of a synthetic androgen, methyltestosterone, to the lithogenic diet induced cholelithiasis in castrated animals (50%). The biles of normal and castrated-hormone supplemented hamsters had cholesterol saturation indices of 1.0 and 1.1, respectively, while the bile of the castrated animals remained unsaturated (0.6). The ratio of cholic acid/chenodeoxycholic acid in bile increased after castration, but returned to normal levels following hormonal supplementation. Biliary cholesterol carriers were separated by ultracentrifugation. Animals in the stone-forming groups (normal and castrated-hormone treated) had a significant proportion of their biliary cholesterol in vesicles (44 and 46%, respectively); castrated hamsters had less cholesterol in vesicle form (9%). The molar ratio of cholesterol/phospholipid in vesicles was reduced after castration (0.93 vs. 0.42) and increased by hormonal supplementation (1.89). In conclusion, when compared to normal male hamsters fed a standard lithogenic diet, castration reduced the cholesterol saturation of bile, lowered the vesicular/micellar ratio in bile, and inhibited cholesterol cholelithiasis. Dietary androgen supplementation increased the lithogenicity of bile, resulting in stone formation in castrated animals.
Lipids 31, 945–948 (1996).

Generally, it is believed that sex hormones influence the incidence of cholesterol cholelithiasis in man (1–4). We have previously shown that male hamsters formed cholesterol gallstones when fed a lithogenic diet (LD), whereas female hamsters fed the identical diet were completely resistant to stone formation (5–7). Dietary administration of the synthetic androgen, methyltestosterone (MeT), to female hamsters induced cholelithiasis to levels observed in males (7). However, the role of the male hormone in gallstone formation remained unclear.

Castration of the male hamster is a surgical procedure which allowed us to evaluate whether male hormones affect cholelithiasis. The dietary addition of the sex hormone to the castrated animal can then be used to study gallstone induction. In this report, we evaluated, for the first time, the effect of (i) castration and (ii) castration with dietary MeT supplementation on biliary lipids, cholesterol carriers in bile, and cholesterol cholelithiasis.

MATERIALS AND METHODS

Animals and diets. Thirty-five male golden Syrian hamsters (four weeks old, 60–74 g) were purchased from Sasco, Inc. (Omaha, NE). The animal protocols were approved by the Institutional Committee on Scientific Activities. Twenty-three hamsters were castrated under anesthesia using an intramuscular injection of ketamine (20 mg/100 g body weight) (Fort Dodge Laboratories, Inc., Fort Dodge, IA). A sham operation was performed on 12 hamsters used as controls. After the operation, the animals were given water and rodent chow *ad libitum* for one week prior to feeding the experimental diets. The animals were fed the following diets for 6 wk: group 1 (control, $n = 12$), lithogenic diet (LD); group 2 (castrated, $n = 11$), LD; group 3 (castrated, $n = 12$), LD + 0.05% MeT. The LD contained 43.6% corn starch, 20.0% casein, 14.6% dyetrose (soluble starch), 10.0% fiber (cellulose), 5.0% salt mix (modified U.S.P. XIV salt mix No. 200951), 4.0% butterfat, 2.0% corn oil, 0.5% vitamin mix (no. 300000), and 0.3% cholesterol (Dyets, Inc., Bethlehem, PA). At the end of the feeding period, the hamsters were fasted overnight and anesthetized by intramuscular injection with 20 mg/100 g body weight of ketamine. After laparotomy, gallbladder bile was aspirated and aliquoted for analysis of biliary lipids and separation of cholesterol carriers. The gallbladder was removed and examined for gallstones.

Analytical procedures. The presence of cholesterol gallstones and crystals in the gallbladder was confirmed by polarizing light microscopy (Olympus MCHAP microscope; Olympus Corp., Lake Success, NY). Bile acids were analyzed by gas–liquid chromatography as the methyl ester acetate derivatives as previously reported (8). The total phospholipid concentration was determined enzymatically (Wako Pure

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Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; CSI, cholesterol saturation index; DCA, deoxycholic acid; EE, ethinylestradiol; LCA, lithocholic acid; LD, lithogenic diet; MeT, methyltestosterone.

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Chemical Industries, Ltd., Osaka, Japan). The cholesterol concentration was determined by gas–liquid chromatography as the trimethylsilylether derivative (9).

The cholesterol carriers were separated by sucrose linear density gradient ultracentrifugation (Ayyad, N., Cohen, B.I., Ohshima, A., and Mosbach, E.H., submitted for publication). Fresh gallbladder bile was centrifuged at 5,000 rpm for 30 min. The supernatant solution was incubated with [1α, 2α (n)-^{3}H] cholesterol and 1-palmitoyl-2-[1-^{14}C] oleoyl phosphatidylcholine (Amersham Life Science, Arlington Heights, IL) under N_2 for one hour at 37°C with shaking. Sucrose (772 mg/mL bile) was dissolved in 50 μL of labeled bile and placed at the bottom of an OptiSeal centrifuge tube (Beckman Instruments Inc., Palo Alto CA). A sucrose linear density gradient (0–46%, 5 mL) was layered on top of the bile sample. The samples were placed in a NVT 90 near vertical rotor (Beckman Instruments Inc., Fullerton, CA) and centrifuged in a L-60 ultracentrifuge (Beckman Instruments Inc.) at 50,000 rpm for one hour. Fractions (total, 30–32) were collected from the bottom of the tube. Twenty μL of each fraction was counted in a Beckman LS 3801 liquid scintillation system with automatic quench correction, using Aquasol-2 (NEN Research Products, Boston, MA) as the scintillation fluid.

Calculations and statistics. Differences in the incidence of cholesterol gallstones were calculated by chi-square (10). The cholesterol saturation index (CSI) was determined as previously reported (11,12). All data are presented as the mean ± SD. The means were compared by analysis of variance to determine the F statistic; Student's *t*-test was applied to those values for which the F statistic was significant ($P < 0.05$) (10).

RESULTS

All hamsters were healthy and gained weight during the experimental feeding period. Table 1 summarizes the incidence of cholesterol gallstones and biliary lipid composition in male hamsters at sacrifice. Sham operated animals (controls, group 1) had a gallstone incidence of 17%. Castrated hamsters formed cholesterol stones only when MeT was added to the diet (group 2 vs. group 3, 0 vs. 50%). Castration decreased biliary cholesterol (group 1 vs. group 2, 6.6 vs. 3.0 mol%) and total biliary lipids (group 1 vs. group 2, 8.5 vs. 5.8 g/dL); these values were restored by MeT supplementation to levels found in the sham group (group 3, cholesterol, 7.4 mol%; total lipids, 9.4 g/dL). In contrast, biliary bile acid was significantly higher in group 2 than groups 1 and 3. Consequently, the CSI in group 2 was significantly lower (0.6) than groups 1 and 3 (1.0 and 1.1, respectively). Table 2 shows the biliary bile acid composition at sacrifice. Chenodeoxycholic acid (CDCA) and lithocholic acid (LCA) were lower in the castrated hamsters (group 2) than in the controls (group 1) and the MeT-supplemented animals (group 3); cholic acid (CA) and deoxycholic acid (DCA) were higher in group 2 than groups 1 and 3. The ratio of CA metabolites to CDCA metabolites in group 2 was significantly higher (3.4) than groups 1 and 3 (1.1 and 1.6, respectively).

Table 3 summarizes cholesterol carriers in bile and the molar ratio of cholesterol to phospholipid in each carrier. Groups 1 and 3 had a higher proportion of vesicles than group 2 (44 and 46% vs. 9%, respectively). Vesicles and micelles in groups 1 and 3 had a significantly higher cholesterol/phospholipid ratio than those in group 2.

DISCUSSION

In the present study, we deprived male hamsters of the sex hormone *via* castration and studied gallstone formation. In contrast to normal males, the bile of castrated hamsters remained unsaturated and no stones developed. Dietary administration of MeT to the castrated animals increased cholesterol saturation and induced stone formation. We have previously showed that the male sex hormone plays an important role in cholesterol cholelithiasis in female hamsters (7). These results indicated that the castrated male and the normal female hamster responded similarly to dietary MeT. We have also found that in the female hamster, dietary administration of MeT increased the secretion of biliary cholesterol while decreasing bile acid secretion and bile flow; these alterations induced lithogenic bile (Ohshima, A., Cohen, B.I., Ayyad, N., and Mosbach, E.H., submitted for publication). In the castrated male hamster, MeT appeared to have a similar effect on the secretion of biliary lipids as observed in the female. Other sex hormones also affect biliary lipid secretion and cholelithiasis: the female hormone, ethinylestradiol (EE), increased the cosecretion mechanism of cholesterol and phospholipid (13) and stimulated the formation of gallstones (14). Another female hormone, progesterone, also increased the secretion of biliary cholesterol (15) and induced cholesterol crystals when admin-

TABLE 1
Incidence of Cholesterol Gallstones and Biliary Lipid Composition at Sacrifice[a]

Group	Castration	MeT	Cholesterol gallstones %	Total lipids g/dL	Bile acid mol%	Phospholipid mol%	Cholesterol mol%	Cholesterol saturation index
1	–	–	2/12 (17)	8.5 ± 2.1	74.2 ± 4.2	19.2 ± 3.1	6.6 ± 1.2	1.0 ± 0.1
2	+	–	0/11 (0)	5.8 ± 2.1[b]	80.4 ± 2.6[b]	16.6 ± 1.9	3.0 ± 1.0[b]	0.6 ± 0.1[b]
3	+	+	6/12 (50)[c]	9.4 ± 2.3[c]	73.7 ± 4.4[c]	19.0 ± 3.4	7.4 ± 1.1[c]	1.1 ± 0.1[b,c]

[a]See the Materials and Methods section for details of animal groups and diets. MeT, methyltestosterone. Values are mean ± SD. [b]Differs from group 1, $P < 0.05$. [c]Differs from group 2, $P < 0.01$.

TABLE 2
Biliary Bile Acid Composition (%) at Sacrifice[a]

Group	CA	CDCA	DCA	LCA	ACA	HDCA	MDCA	$CA^+/CDCA^+$
1	35.0 ± 4.0	32.3 ± 4.5	11.2 ± 3.2	2.6 ± 0.7	3.0 ± 1.6	9.4 ± 2.6	2.8 ± 0.8	1.1 ± 0.3
2	53.5 ± 4.0[b]	13.5 ± 1.9[b]	17.1 ± 2.1[b]	1.2 ± 0.8[b]	1.8 ± 0.9	5.2 ± 2.0[b]	1.7 ± 0.3[b]	3.4 ± 0.7[b]
3	45.4 ± 2.9[b,c]	28.6 ± 4.7[c]	11.5 ± 2.8[c]	2.6 ± 1.1[c]	3.2 ± 1.4[c]	6.1 ± 3.2[b]	1.8 ± 0.5[b]	1.6 ± 0.4[b,c]

[a]CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; ACA, allocholic acid; HDCA, hyodeoxycholic acid; MDCA, murideoxycholic acid; $CA^+/CDCA^+$, ratio of CA metabolites (CA, ACA, DCA) to CDCA metabolites (CDCA, LCA, HDCA, MDCA). Values are mean ± SD. [b]Differs from group 1, $P < 0.02$. [c]Differs from group 2, $P < 0.04$.

istered with EE (16). Sex hormones (androgen, estrogen, and gestagen) might have similar stimulatory effects on biliary cholesterol secretion and stone formation.

The biliary bile acid composition differed in male and female hamsters: females had a higher CA/CDCA ratio than males (17). In a previous study, MeT altered the bile acid composition of female hamsters to that found in males (7). We found that castration increased CA metabolites and decreased CDCA metabolites in the bile of male hamsters; MeT supplementation restored the bile acid composition to that of the control males. Androgens might decrease the activity of the hepatic steroid 12α-hydroxylase, which determines the ratio of CA/CDCA (17). Since CDCA stimulates biliary cholesterol secretion more than CA in hamsters (18), the change in bile acid composition (decreased CA/CDCA ratio) could be responsible, at least in part, for the increase of biliary lithogenicity in normal male or androgen supplemented animals.

Castration reduced the proportion of vesicles in bile and prevented stone formation, while MeT increased this cholesterol carrier to control levels and stimulated lithiasis. These results support the hypothesis that vesicle formation precedes cholesterol gallstone formation (19–21). The amount of vesicles in the control and the castrated-MeT treated hamsters were similar; however, the cholesterol/phospholipid ratio of these vesicles was higher in the MeT treated group (0.93 vs. 1.89, respectively). Vesicles having a higher cholesterol/phospholipid ratio tend to be less stable and form cholesterol crystals more rapidly (20–22). In our experiment, castrated-MeT treated hamsters formed more stones than the control animals; even though the CSI of both groups was similar and above unity. There is a general agreement that cholesterol supersaturation is necessary but not sufficient for cholesterol gallstone formation (6,22–26). These results suggest that the cholesterol/phospholipid ratio of vesicles may be a more rigorous parameter to predict cholelithiasis than the CSI.

TABLE 3
Distribution of Cholesterol Between Its Carriers in Bile[a]

Group (*n*)[a]	Vesicles % CH	Micelles % CH	CH/PL in vesicles	CH/PL in micelles
1 (3)	44 ± 4	56 ± 4	0.93 ± 0.10	0.23 ± 0.01
2 (4)	9 ± 4[b]	91 ± 4[b]	0.42 ± 0.11[b]	0.17 ± 0.03[b]
3 (4)	46 ± 4[c]	54 ± 4[c]	1.89 ± 0.70[c]	0.25 ± 0.02[c]

[a]Biliary cholesterol carriers were separated by ultracentrifugation using a sucrose linear density gradient. See the Materials and Methods section for details. CH/PL, molar ratio of cholesterol to phospholipid. Values are mean ± SD. (*n*) Refers to the number of samples analyzed in each group.
[b]Differs from group 1, $P < 0.03$.
[c]Differs from group 2, $P < 0.04$.

In conclusion, castration resulted in unsaturated bile, reduction of total biliary lipids, and inhibition of cholesterol cholelithiasis in male hamsters. Dietary supplementation with MeT resulted in elevation of biliary lithogenicity and gallstone formation. The proportion of cholesterol in vesicles as well as the cholesterol/phospholipid ratio in vesicles was markedly reduced by castration but increased after hormonal supplementation. These results suggest that there is a strong relationship between hormonal factors and cholesterol gallstone formation.

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Dietary Fat Alters Biliary Lipid Secretion in the Hamster

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ABSTRACT: Dietary fat has been found to alter the incidence of cholesterol gallstones in hamsters: butterfat intensifies while safflower oil reduces lithiasis. We now report how dietary fat affects bile flow and biliary lipid secretion in this model. Male hamsters were fed one of three experimental diets: a control diet (containing 0.3% cholesterol); control diet + 4.0% butterfat; or control diet + 4.0% safflower oil. After three weeks, bile samples were collected *via* an external biliary fistula. The endogenous bile acid pool was depleted for 120 min followed by increasing rates of taurocholate infusion for 160 min. Basal secretion of biliary lipids was measured during the bile acid depletion period. Basal bile flow and bile acid output were not significantly different in the three groups. Dietary butterfat increased basal cholesterol output compared to the control diet (0.037 vs. 0.025 μmol/min · kg, respectively); safflower oil did not change cholesterol output (0.027 μmol/min · kg). Hamsters fed butterfat or safflower oil secreted more phospholipid (0.171 and 0.178 μmol/min · kg, respectively) than controls (0.131 μmol/min · kg). The cholesterol/phospholipid output ratio of the butterfat group was higher than the safflower oil group (0.220 vs. 0.153, respectively). Effects of dietary fat on several relationships between bile flow and biliary lipid secretion were analyzed by linear regression using the data for the entire bile collection period (bile acid depletion and taurocholate infusion). Butterfat and safflower oil did not change either bile acid dependent or bile acid independent bile flow. Hamsters fed butterfat had a higher linkage coefficient (slope) of cholesterol vs. bile acid output than the safflower oil group (0.023 vs. 0.009, respectively). The linkage coefficient of phospholipid vs. bile acid output of the butterfat group was higher than the controls (0.278 vs. 0.185, respectively). In summary, butterfat induced a high cholesterol and phospholipid secretion with a high cholesterol/phospholipid output ratio; safflower oil induced a high phospholipid secretion with a low cholesterol/phospholipid output ratio. Butterfat and safflower oil have different effects on biliary lipid secretion. These differences in biliary lipid secretion may explain, in part, how butterfat and safflower oil differ in affecting gallstone formation in hamsters.

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It has been shown that different nutrients affect cholesterol cholelithiasis (1). In our laboratory, we have found that dietary fat and fatty acids alter the incidence of cholesterol gallstones in hamsters (2–4). Addition of 4.0% butterfat to a purified diet containing 0.3% cholesterol increased the incidence of cholesterol gallstones after 6 wk (63–84%); 4.0% safflower oil reduced cholelithiasis (13–22%) (3,5–7). Values observed for the biliary cholesterol saturation indices (greater than unity) and the amount of biliary cholesterol in vesicles were similar and could not explain the differences in stone formation (5–7). Biliary lipid secretion is a dynamic measurement of the output of cholesterol, phospholipid, and bile acids (8,9), which may enable us to explain some of the early events of cholelithiasis. We now report, for the first time, the effects of dietary butterfat or safflower oil on bile flow and biliary lipid secretion in our hamster model.

MATERIALS AND METHODS

Animals and diets. Male golden Syrian hamsters (50–55 g) were purchased from Sasco, Inc. (Omaha, NE). The animal protocols were approved by the Institutional Committee on Scientific Activities. The hamsters were divided into three groups and fed the experimental diets and water *ad libitum* for three weeks prior to surgery: group 1 ($n = 6$), purified diet + 0.3% cholesterol (control diet); group 2 ($n = 5$), control diet + 4.0% butterfat; group 3 ($n = 5$), control diet + 4.0% safflower oil. The purified diet contained 45.6% corn starch, 20.9% casein, 15.3% dyetrose (soluble starch), 10.4% fiber (cellulose), 5.2% salt mix (modified U.S.P. XIV salt mix No. 200951), 2.1% corn oil (source of essential fatty acids), and 0.5% vitamin mix (no. 300000) (Dyets, Inc., Bethlehem, PA).

Surgical procedures. At the end of the feeding period, the hamsters were anesthetized by intraperitoneal injection of 20 mg/100 g body weight of ketamine (Ketaset; Fort Dodge Laboratories, Inc., Fort Dodge, IA). Additional intramuscular injections of ketamine (10 mg) were used to maintain anesthesia. A polyethylene catheter (PE-10, 0.28 mm i.d.; Clay Adams, Parsippany, NJ) was inserted into the femoral vein, and 0.9% saline was infused at a rate of 1.13 mL/h using a Harvard syringe pump (Harvard Apparatus, Millis, MA). After laparotomy, the cystic duct was ligated with a hemostatic clip, and the common bile duct was cannulated using poly-

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Abbreviations: LKC, linkage coefficient; TC, sodium taurocholate.

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ethylene tubing (PE-10). Saline infusion was maintained for two hours in order to deplete the endogenous bile acid pool. Sodium taurocholate (TC) (Sigma Chemical Co., St. Louis, MO) was then infused at increasing rates of 6 and 18 μmol/h/100 g body weight together with trace amounts of [24-^{14}C] TC (Amersham Life Science, Arlington Heights, IL); each dose of TC was infused for 80 min. Bile samples were collected every 20 min in preweighed vials for the entire period (280 min). Each sample was weighed, divided into aliquots, and analyzed for biliary lipids. At the end of the experiment, the gallbladder was removed and examined for gallstones using polarizing light microscopy (2).

Analytical procedures. Bile volumes were determined gravimetrically. The recovery of [24-^{14}C]TC in 10 μL of each hepatic bile sample was quantitated using a Beckman LS 3801 liquid scintillation system (Beckman Instruments, Fullerton, CA) with automatic quench correction (5). Total bile acids, cholesterol, and phospholipids were each measured enzymatically using commercial kits (bile acid, # 450-A and cholesterol, # 352-20; Sigma Chemical Co.; phospholipid, Wako Pure Chemical Industries, Ltd., Osaka, Japan) (2).

Calculations and statistics. Bile flow is expressed as microliters per minute per kilogram body weight; biliary lipid output is expressed as micromoles per minute per kilogram body weight. Since bile flow and biliary lipid secretion were variable during the first 40 min, the basal bile flow and biliary lipid output during depletion of the endogenous bile acid pool were calculated using the data for the 40–120 min period before TC infusion (three to four samples per animal). The data are presented as the mean ± SD. The means were compared by analysis of variance to determine the F statistic; Student's *t*-test was applied to those values for which the F statistic was significant ($P < 0.05$) (10). The relationships between bile flow vs. bile acid output, cholesterol output vs. bile acid output, phospholipid output vs. bile acid output, and cholesterol output vs. phospholipid output were analyzed by linear regression for the bile acid depletion and TC infusion periods (ten to twelve samples per animal) (8,9). The regression lines were plotted, and the slopes and *y*-intercepts were calculated. The linkage coefficient (LKC) is defined as the slope of the regression line, that is, the increment in cholesterol or phospholipid output induced per increment of bile acid output, or the incremental ratio of cholesterol output to phospholipid output (8,9). Comparisons of slopes or *y*-intercepts were made using SAS/STAT (version 6.10; SAS Institute Inc., Cary, NC) (11,12). A difference was considered significant at $P < 0.05$.

Some hamsters had normal bile flow, bile acid output, and cholesterol output, but their phospholipid output was significantly lower than normal. In these animals, the ratio of cholesterol/phospholipid output was considerably higher (1.0–10.0) than in normal hamsters (0.1–0.3) during both the bile acid depletion and TC infusion periods. Consequently, data obtained from these animals were excluded from the analyses (one hamster from group 1, two from group 2, and one from group 3). There were a sufficient number of samples to compare the results statistically, since we collected as many as twelve samples per animal.

RESULTS

All hamsters remained healthy and gained weight during the experiment. At the end of the feeding period, surgery was performed to establish a biliary fistula and to insert a femoral vein cannula. TC was infused i.v. at increasing doses after depletion of the endogenous bile acid pool. When the maximum dose of TC (18 μmol/h/100 g body weight) was infused, the recovery of labeled TC was 98.5 ± 4.4%, 96.8 ± 5.4%, and 91.4 ± 10.1% for groups 1–3, respectively. After bile collection, the gallbladder was examined for the presence of cholesterol gallstones. No stones were detected in any of the animals after 3 wk.

Table 1 summarizes basal bile flow, biliary lipid output, and the ratio of cholesterol output to phospholipid output during the depletion of the endogenous bile acid pool. Basal bile flow and bile acid output did not differ among the three groups. Dietary safflower oil did not change basal cholesterol output (group 1 vs. group 3, 0.025 vs. 0.027 μmol/min · kg); butterfat induced a higher basal cholesterol output (group 2, 0.037 μmol/min · kg) than the control and safflower oil diet. Butterfat and safflower oil increased basal phospholipid output (groups 2 and 3, 0.171 and 0.178 μmol/min · kg) as compared to the control diet (group 1, 0.131 μmol/min · kg). Group 2 had a higher ratio of cholesterol output to phospholipid output than group 3 (0.220 vs. 0.153, respectively).

TABLE 1
Basal Biliary Lipid Secretion During Depletion of the Endogenous Bile Acid Pool[a]

Group # (*n*)[b]	Bile flow (μL/min · kg)	BA output (μmol/min · kg)	CH output (μmol/min · kg)	PL output (μmol/min · kg)	Ratio of CH output/PL output
1 (18)	65.0 ± 6.2	0.224 ± 0.083	0.025 ± 0.010	0.131 ± 0.037	0.181 ± 0.044
2 (12)	65.5 ± 8.5	0.204 ± 0.085	0.037 ± 0.013[c]	0.171 ± 0.050[c]	0.220 ± 0.068
3 (15)	59.2 ± 18.3	0.233 ± 0.043	0.027 ± 0.010[d]	0.178 ± 0.048[c]	0.153 ± 0.030[d]

[a]Data were used for the 40–120-min period before taurocholate infusion. See the Materials and Methods section for details of statistical analysis. Group 1, purified diet + 0.3% cholesterol (control diet); group 2, control diet + 4.0% butterfat; group 3, control diet + 4.0% safflower oil. BA, bile acid; CH, cholesterol; PL, phospholipid.
[b](*n*), Number of bile samples. Three to four samples were obtained from each animal.
[c]Differs from group 1; $P < 0.05$.
[d]Differs from group 2; $P < 0.05$.

TABLE 2
Coefficients of Linear Regression Equations for Relationships Between Bile Flow, Bile Acid, Cholesterol, and Phospholipid Outputs[a]

Group	Bile flow vs. BA output			CH output vs. BA output			PL output vs. BA output			CH output vs. PL output		
# (*n*)[b]	Slope[c]	Intercept[d]	*r*	Slope[e]	Intercept	*r*	Slope[e]	Intercept	*r*	Slope[e]	Intercept	*r*
1 (57)	29.5	63.9	0.87	0.017	0.018	0.59	0.185	0.093	0.88	0.117	0.005	0.85
2 (36)	33.1	61.5	0.85	0.023	0.026[f]	0.71	0.278[f]	0.102	0.95	0.081[f]	0.018[f]	0.74
3 (46)	25.3	58.7	0.49	0.009[g]	0.023	0.37	0.224	0.114	0.80	0.064[f]	0.012[f]	0.76

[a]Coefficients were calculated from linear regression lines plotted using data for the bile acid depletion and taurocholate infusion periods. See the Materials and Methods section for details of statistical analysis. BA, bile acid; CH, cholesterol; PL, phospholipid; *r*, correlation coefficient.
[b](*n*), Number of bile samples. Ten to twelve samples were obtained from each animal.
[c]Defined as bile acid dependent bile flow (μL/μmol of bile acid).
[d]Defined as bile acid independent bile flow (μL/μmol of bile acid).
[e]A linkage coefficient, defined as increment of lipid output induced per increment of lipid output.
[f]Differs from group 1; $P < 0.04$.
[g]Differs from group 2; $P < 0.01$.

Table 2 lists the coefficients of the linear regression equations for several relationships between bile flow and biliary lipid outputs. The regression line of bile flow vs. bile acid output showed that both dietary butterfat and safflower oil did not change either bile acid-dependent (slope) or bile acid-independent (*y*-intercept) bile flow. The relationship between cholesterol vs. bile acid output showed that butterfat induced a higher LKC than safflower oil (group 2 vs. group 3, 0.023 vs. 0.009) (Fig. 1). The LKC for controls (group 1) was 0.017; this value was increased 35% by butterfat and decreased 47% by safflower oil. The LKC of phospholipid vs. bile acid output was also altered by dietary fat (Fig. 2): the slope of phospholipid vs. bile acid was 50% higher in the butterfat group than the control group (group 2 vs. group 1, 0.278 vs. 0.185). The LKC for safflower oil (group 3, 0.224) was intermediate between groups 1 and 2. The LKC of cholesterol vs. phospholipid output for group 1 (0.117) was higher than that for groups 2 and 3 (0.081 and 0.064, respectively); the *y*-intercept for group 1 (0.005) was lower than those of groups 2 and 3 (0.018 and 0.012, respectively).

DISCUSSION

The incidence of cholesterol gallstones has been found to change with the type and amount of dietary fat (2–4). In some cases, animals with or without stones had supersaturated bile as well as a significant portion of biliary cholesterol in cholesterol/phospholipid vesicles (5–7). It appeared that supersaturation of bile as well as the presence of cholesterol-rich vesicles are necessary, but are not the only conditions for cholesterol gallstone formation (13–15). The reason for the differences in the incidence of cholelithiasis remained unclear. Measurements of biliary lipid secretion may be useful to examine the early stages of cholelithiasis (16). The precise relationship between dietary fat, biliary lipid secretion, and gallstone formation are not known in our model. Consequently, we now examined the effect of dietary butterfat or safflower oil on bile flow and biliary lipid secretion in bile fistula hamsters. Our standard gallstone induction period is 6 wk (3–7). A 3-wk feeding period was used in this study to examine changes in biliary lipid secretion prior to gallstone formation.

Dietary butterfat increased both basal cholesterol and phospholipid outputs and resulted in a higher cholesterol/phospholipid output ratio compared to controls. Safflower oil stimulated basal phospholipid output and resulted in a lower cholesterol/phospholipid output ratio relative to controls. These results suggested that the reduced lithiasis observed after 6 wk with safflower oil could be related to a lower cholesterol/phospholipid output ratio during the first 3 wk. Butterfat raised the LKC for cholesterol vs. bile acid while safflower oil reduced it relative to controls. Butterfat increased the LKC for phospholipid vs. bile acid; safflower oil also increased this LKC but to a lesser extent. It is generally accepted that cholesterol and phospholipid are secreted together as vesicles from the canalicular membrane of hepatocytes (15,17,18). These results also suggest that fat may affect the secretory relationship between cholesterol and phospholipid.

The dietary fats used in this study had a different fatty acid composition. Butterfat is rich in saturated fatty acids (palmitic acid, 16:0, 27%); safflower oil contains mostly unsaturated fatty acids (linoleic acid, 18:2, 78%) (19). We have reported that palmitic acid and linoleic acid affect cholesterol gallstone incidence in a manner similar to butterfat and safflower oil, respectively (3,4,20). The changes observed in biliary lipid secretion in the present study might be related to the fatty acid composition of the dietary fat.

It has been found that a high-fat diet can stimulate biliary lipid secretion (21). In our experiment, animals fed either butterfat or safflower oil (total fat 6%) secreted more phospholipid than the controls (total fat 2%). However, cholesterol secretion was stimulated only in the butterfat group. Theoretically, dietary fat could affect the biliary cholesterol/phospholipid output ratio by changing one or more of the following factors: (i) secretion or composition of biliary bile acids, (ii) bile acid independent bile flow, and/or (iii) biliary phospholipid composition. First, it has been shown that biliary cholesterol and phospholipid secretions were stimulated by bile acids in a dose-dependent manner; this increase was dependent on the structure of the administered bile acid (9,22). However, in the current experiment, butterfat and safflower oil did not alter biliary bile acid secretion; neither fat changed

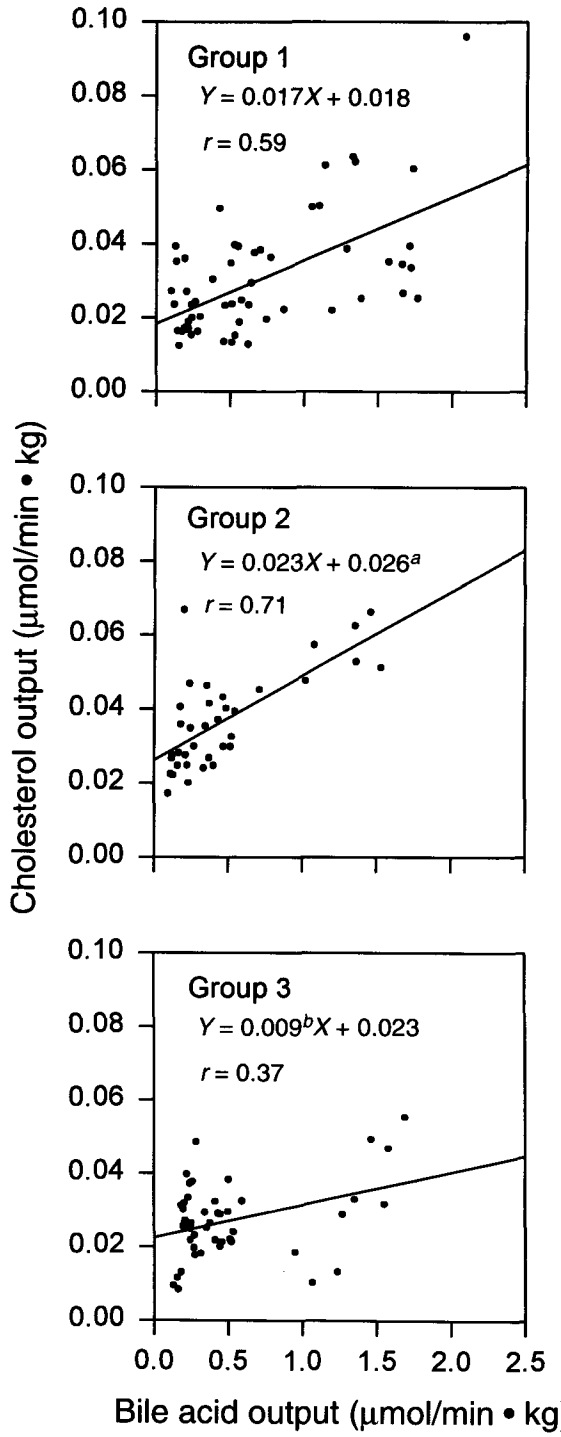


FIG. 1. Relationship between cholesterol output and bile acid output during bile acid depletion and sodium taurocholate infusion periods (6–18 µmol/h · 100 g body weight). Group 1, purified diet + 0.3% cholesterol (control diet); group 2, control diet + 4.0% butterfat; group 3, control diet + 4.0% safflower oil. Coefficients of the linear regression equations were compared among experimental groups. [a]Differs from group 1; $P < 0.04$. [b]Differs from group 2; $P < 0.01$.

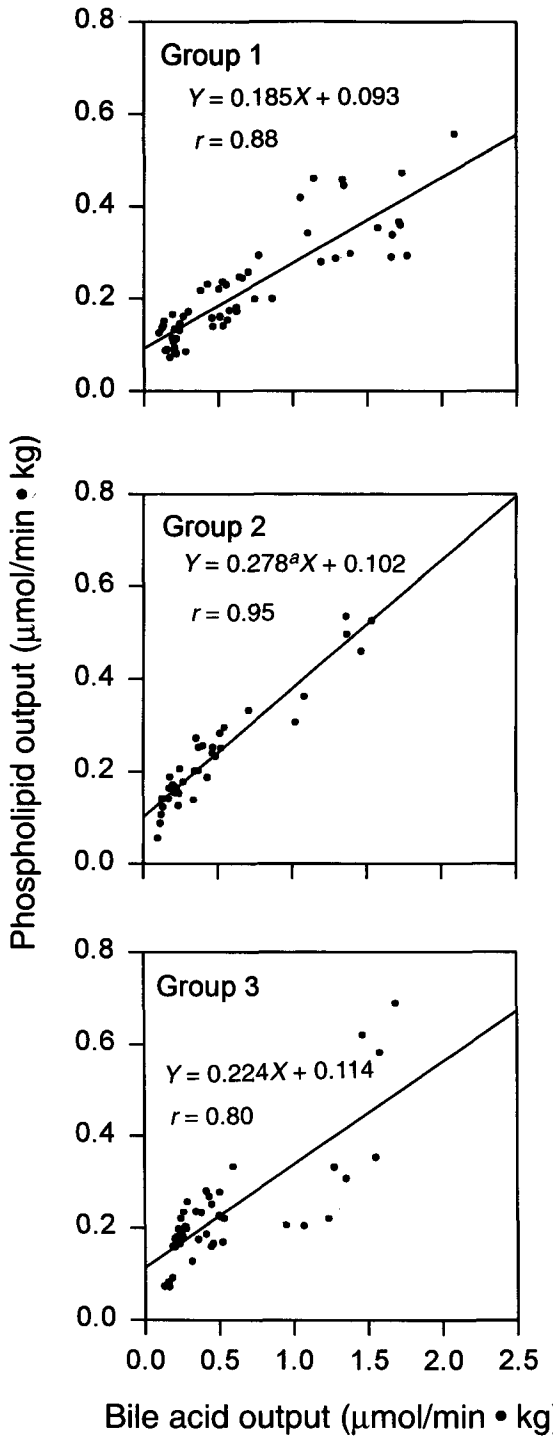


FIG. 2. Relationship between phospholipid output and bile acid output during bile acid depletion and sodium taurocholate infusion periods (6–18 µmol/h · 100 g body weight). Group 1, purified diet + 0.3% cholesterol (control diet); group 2, control diet + 4.0% butterfat; group 3, control diet + 4.0% safflower oil. Coefficients of the linear regression equations were compared among experimental groups. [a]Differs from group 1; $P < 0.04$.

the composition of biliary bile acids (Ref. 5; Ayyad, N., Cohen, B.I., Ohshima, A., and Mosbach, E.H., submitted for publication). Thus, secretion and composition of biliary bile acids were not factors in changing the cholesterol and phospholipid output. Second, it is known that the bile acid independent bile flow was another regulatory factor that could af-

fect the biliary secretion of cholesterol and phospholipid: a lower bile acid independent bile flow induced a more effective secretion of these lipids (23). In our study, butterfat or safflower oil did not change the bile acid independent bile flow, suggesting that this factor was not responsible for the changes in the biliary lipid secretion. Third, it was shown that the fatty acid components of the biliary phospholipids reflected the composition of the exogenous fat (7,20,24,25). We have previously found that dietary safflower oil and 18:2 increased the 16:0–18:2 phosphatidylcholine molecular species in bile by approximately 25% (20). Robins *et al.* (26) showed that the various molecular species of biliary phosphatidylcholine altered the amount of cholesterol transported into bile. It is possible that the changes we observed in biliary phospholipids may also alter the cholesterol/phospholipid output ratio. Furthermore, the changes observed in the phosphatidylcholine molecular species could also affect the stability of vesicles from which cholesterol crystals nucleate: unsaturated fatty acids stabilize vesicles to a greater extent than saturated fatty acids (27–30).

Many studies have examined the effects of unsaturated fat on bile and plasma cholesterol levels (24,25,31,32). It was reported that safflower oil caused an increase in biliary cholesterol secretion (24,25,31,32). This result was opposite from that reported in the present study. However, we cannot compare these experiments to our study because of major differences in the experimental design. These earlier studies employed a significantly lower level of exogenous cholesterol (0.02%) to maintain normal cholesterol synthesis; our experiments used 0.3% cholesterol to induce gallstones, a dose which eliminated endogenous cholesterol synthesis (33). The amount of exogenous cholesterol appears to modify the effect of dietary fat on biliary cholesterol secretion.

In conclusion, dietary administration of butterfat or safflower oil had different effects on the secretion of biliary cholesterol and phospholipid which could explain, in part, their role in cholesterol cholelithiasis in hamsters.

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Effects of Dietary Heated Fats on Rat Liver Enzyme Activity

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

Lipids 31, 955–962 (1996).

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

Food fried in test oil[b] and treatment

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

RESULTS

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Rats pair-fed graded doses of 7-DH diets showed a highly significant increase in Cyt.b_5 when animals were fed the 15%

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

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

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

7-DH in comparison with the control group of rats fed the NH diet. When the 10% 7-DH was fed, the increased value was significant at $P < 0.01$ when compared to the control animals (Table 5).

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

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

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

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

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

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

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

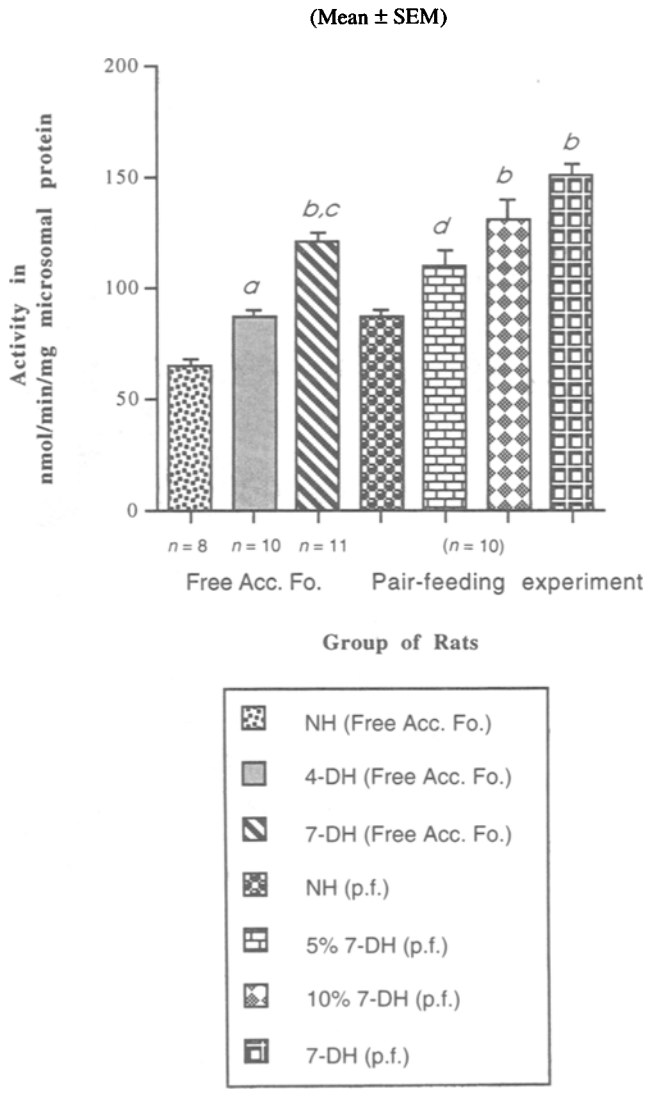


FIG. 1. NADPH-cytochrome P_{450} reductase activity in liver microsomes of rats fed NH, 4-DH, and 7-DH diets. NH, control group of rats fed diet containing nonheated (NH) partially hydrogenated soybean oil (PHSBO); 4-DH, group of rats fed diet containing PHSBO used 4 d (4-DH) for frying foodstuffs; 7-DH,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

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

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

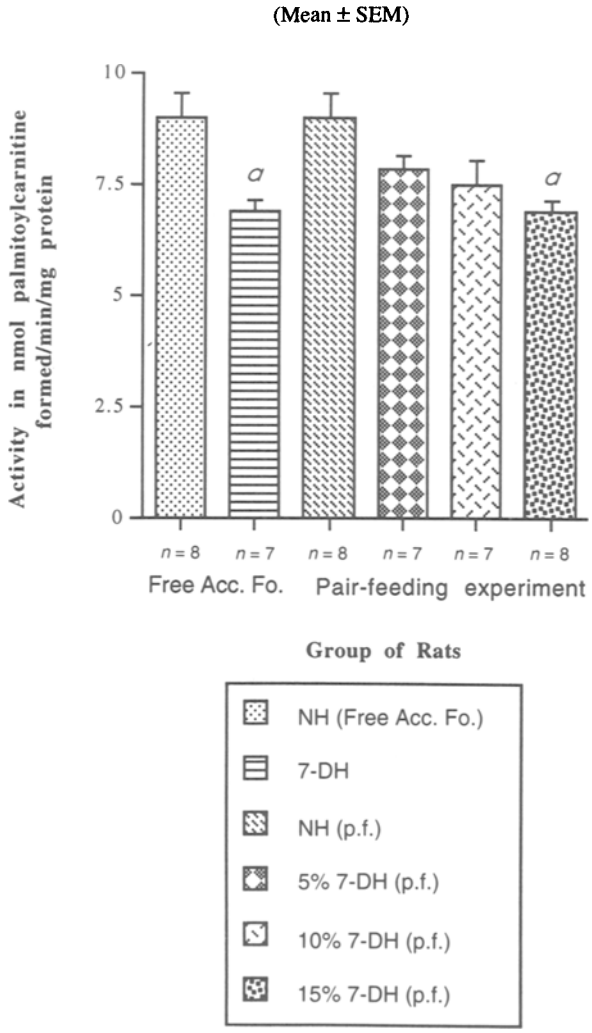


FIG. 2. Carnitine palmitoyltransferase-I activity in liver mitochondria of rats fed NH and 7-DH diets. Abbreviations and legend descriptions as in Figure 1. $^aP < 0.01$ compared to the control group of rats fed the NH diet.

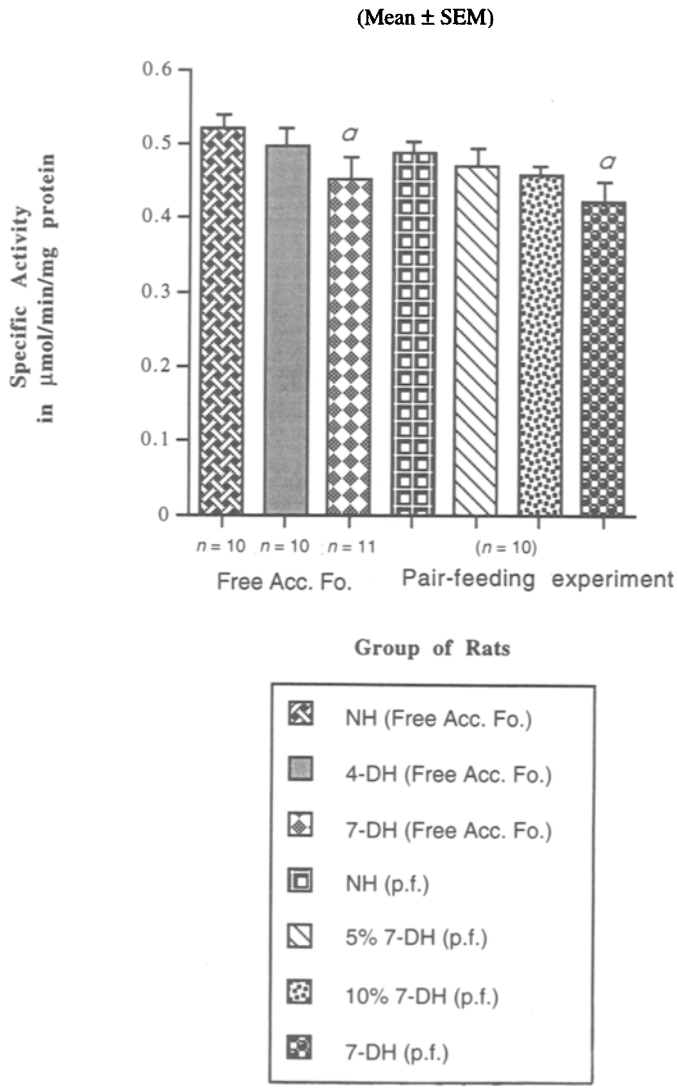


FIG. 3. Isocitrate dehydrogenase activity in liver of rats fed NH, 4-DH, and 7-DH diets. $^aP < 0.05$ when the 7-DH diet group of rats was compared to the NH group of rats. See Figure 1 for abbreviations and legend descriptions.

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

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

DISCUSSION

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

Feed efficiency data were not significantly different among groups fed either diets in the free access to food or by pair-feeding experiments, suggesting that the effects observed in

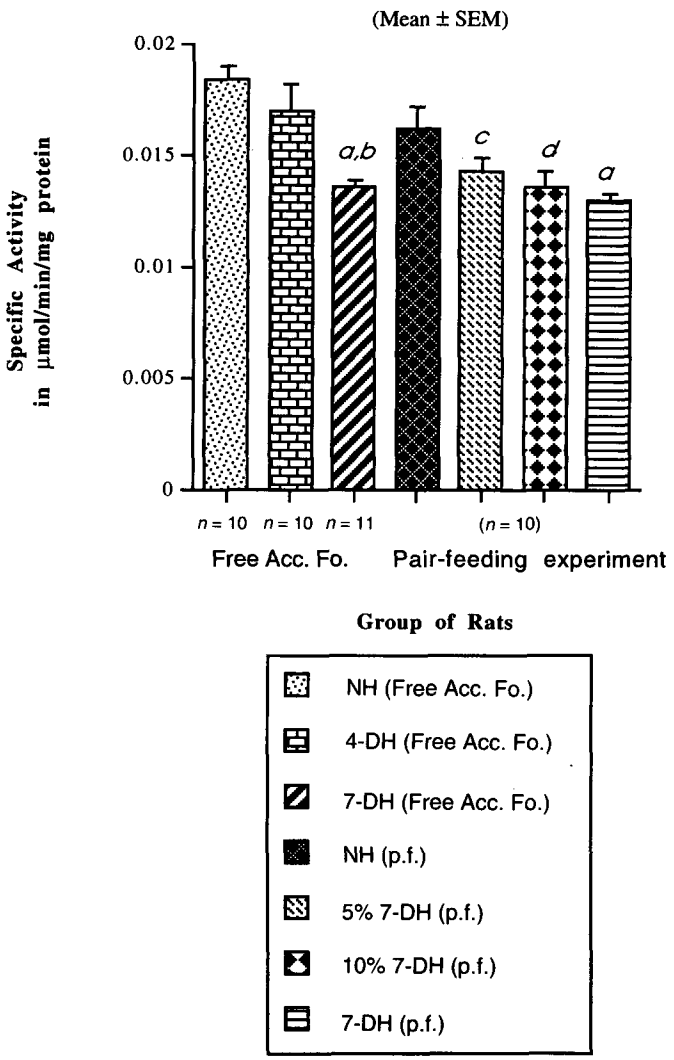


FIG. 4. Glucose 6-phosphate dehydrogenase activity in liver of rats fed NH, 4-DH, and 7-DH diets. See Figure 1 for abbreviations and legend descriptions. [a]$P < 0.0001$ when the 7-DH diet group of rats was compared to the NH group of rats. [b]$P < 0.0001$ when the 7-DH diet group of rats was compared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

this study cannot be attributed to the amount of diet ingested by the rats and may simply reflect the content of the oils fed to animals.

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

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

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

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

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

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

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

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

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

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

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protect against *in vitro* liver lipid peroxidation, whereas further supplementation to high levels do not bring additional protection. Relative percentage reduction of fatty acid unsaturation in all kinds of membrane phospholipids was found both in animals receiving low or high vitamin E dietary levels compared with those receiving the medium level.

MATERIALS AND METHODS

Animals, diets, and vitamin E. Dunkin-Hartley male guinea pigs weighing 200–250 g were obtained from IFFA-CREDDO (Lyon, France). Three diets differing in vitamin E content were prepared by U.A.R. (Perpignan, France) by adding d,l-α-tocopherol acetate to the standard guinea pig diet (diet 114; U.A.R.). The vitamin E dietary content was 1,500 mg of vitamin E per kg diet (group HIGH), 150 mg vitamin E/kg (group MEDIUM), or 15 mg vitamin E/kg (group LOW). These vitamin E contents were confirmed after analysis by high-performance liquid chromatography (HPLC). Briefly, tissue samples (20–40 mg) were saponified in the presence of 0.2 mL of 15% ascorbic acid, 2 mL of ethanol containing 0.025% butylated hydroxytoluene, and 1 mL of 40% potassium hydroxide. Tocopherol was then extracted with hexane containing 0.025% butylated hydroxytoluene, the hexane was removed with N_2, the residue was redissolved in 0.5 mL ethanol, and 20 μL were injected in the HPLC. Mobil phase was 100% methanol, and the column was a C_{18} reverse phase Machery Nagel (Duren, Germany) 4.6 × 100 mm, 7 μm. The system was composed of a Waters 510 pump (Millipore Corporation, Milford, MA), a Gilson ultraviolet detector (Middleton, WI) set at 292 nm, and a Spectraphysics integrator (San Jose, CA). The basal diet contained 18.5% protein, 2.9% fat, 46.9% carbohydrates, 8.4% mineral mix, 1.4% vitamin mix, 11% humidity, and 10.9% nonnutritive bulk. The content of minerals and vitamins per kg of diet was: phosphorus, 8.6 g; calcium, 10.6 g; potassium, 12.0 g; sodium, 3.45 g; magnesium, 3.13 g; manganese, 100 mg; iron, 320 mg; copper, 26 mg; zinc, 85 mg; cobalt, 1.61 mg; vitamin A, 19,000 I.U.; vitamin D_3, 2,031 I.U.; thiamine, 22.5 mg; riboflavin, 21 mg; pantothenic acid, 123 mg; pyridoxine, 10.7 mg; menadione, 55 mg; niacin, 193 mg; folic acid, 7.3 mg; biotin, 0.275 mg; choline, 1,740 mg; myo-inositol, 250 mg; vitamin B_{12}, 0.054 mg; *p*-aminobenzoic acid, 10 mg; and vitamin C, 660 mg. This amount of dietary vitamin C was found to be optimal from the point of view of oxidative stress and fatty acid composition in a previous study (12) in the liver of male guinea pigs of the same age than those used here.

The animals were fed for five weeks the three experimental diets, and they were maintained inside aseptic air positive-pressure animal cabinets (A 130 SP; Flufrance, Cachan, France) equipped with an HEPA air filter (99.999% for particles >0.3 μm) at the inlet. At the end of the dietary regimen, the animals were sacrificed by decapitation and liver samples were immediately excised and stored at –80°C.

Nonenzymatic in vitro *lipid peroxidation.* Sensitivity to nonenzymatic *in vitro* lipid peroxidation was estimated by incubating supernatants of liver samples, obtained by centrifugation at 3,500 × *g* of liver homogenates (prepared in 50 mM phosphate buffer pH 7.4), in the presence of 0.4 mM ascorbate and 0.05 mM $FeSO_4$ for 0, 90, and 180 min at 37°C. At the end of the incubation period, the thiobarbituric acid (TBA) assay was performed by a method specially adapted to tissue extracts (13). MDA (malondialdehyde–bisdimethyl acetal; Merck, Darmstadt, Germany) was used as a standard. The results were expressed as nanomoles of MDA equivalents produced per min per g tissue and represent the sensitivity of liver lipids to an oxygen radical induced oxidative stress.

Oxidative stress in the hydrophilic compartment. In order to estimate the GSH/GSSG ratio, liver samples were homogenized in 5% trichloroacetic acid (TCA) with 0.01 N HCl, and total glutathione was measured by the spectrophotometric recycling assay (14) in the presence of 5,5′-dithiobis (2-nitrobenzoic acid), NADPH, and glutathione reductase at 412 nm. GSSG was assayed by the same method by direct derivatization of GSH homogenizing another sample from the same tissue in the presence of 12.5 mM *N*-ethylmaleimide, followed by alkaline hydrolysis of *N*-ethylmaleimide (15). GSH values were obtained after subtracting GSSG from total glutathione.

Protein carbonyl content was determined spectrophotometrically according to the 2,4-dinitrophenylhydrazine (DNPH) method (16) as previously described (12). Briefly, liver samples were treated during 15 min with 0.1% digitonin, 1 mM EDTA, phenylmethanesulfonyl fluoride (40 μg/mL), and protease inhibitors, centrifuged, the DNA was removed with 1% streptomycin (15-min incubation), and samples were centrifuged again. The supernatants were divided into two portions which were treated with 2 M HCl or 10 mM DNPH in 2 M HCl. Proteins were precipitated with 20 and 10% TCA and the pellets were washed three times with ethyl acetate/ethanol. Pellets were dissolved in 6 M guanidine hydrochloride in 20 mM potassium phosphate (pH 2.3). Each DNPH sample was scanned from 320 to 410 nm against the HCl corresponding sample, and the peak absorbance was used to calculate protein carbonyls (extinction coefficient 22,000 M^{-1} cm^{-1}). The protein concentration was read at 280 nm in the HCl sample using 6 M guanidine hydrochloride as a blank.

Fatty acid analyses. Liver fatty acids were analyzed as previously described (17). Lipids were extracted with 2-propanol that was then evaporated with N_2. The lipid fractions were separated by thin-layer chromatography on silica gel plates. The phospholipid composition was studied using chloroform/methanol/acetic acid/water (50:25:8:4, by vol) and visualized with 10% $CuSO_4$ in 8% H_3PO_4. Lipids were transesterified with 5% HCl in methanol at 75°C. The esters were extracted with *n*-pentane and saturated NaCl, evaporated under N_2 and redissolved in CS_2. Fatty acid methyl esters were analyzed on a gas chromatograph equipped with a fused silica capillary column (Supelcowax TM 10; 30 m × 0.53 mm; Supelco, Inc., Bellefonte, PA). Chromatographic conditions: carrier gas, N_2, at 60 mL/min; flame-ionization

detector with H_2 at 50 mL/min and air at 500 mL/min; injector and detector temperature, 250°C; column temperature was increased from 135 to 195°C: increase of 4° per min; from 195 to 215°C: increase of 1° per min; from 215 to 235°C: increase of 2° per min. Identification of esters was made by comparison with authentic standards (Sigma, St. Louis, MO).

Statistical analysis. Comparisons between the three vitamin E groups were analyzed by one-way analysis of variance (ANOVA). After the ANOVA, the Fisher's least significant difference test was used to analyze significance between paired groups when necessary. *In vitro* lipid peroxidation was analyzed by two-way ANOVA (for time and vitamin E group) and by one-way ANOVA (as a function of incubation time). The 0.05 level was selected as the point of minimal statistical significance in every comparison.

RESULTS

Vitamin E supplementation. Vitamin E supplementation did not affect the growth rate of the animals after either 3 or after 5 wk of treatment (data not shown). The same was true for food ingestion (data not shown). Vitamin E dietary supplementation effectively led to very different hepatic levels of vitamin E in the three groups after 5 wk of supplementation. Increasing vitamin E dietary content one order of magnitude from group LOW to group MEDIUM resulted in a 129% increase in liver α-tocopherol concentration (from 11.9 ± 1.9 to 27.2 ± 3.2 μg/g of tissue). When dietary vitamin E was further increased another order of magnitude from group MEDIUM to group HIGH, the liver α-tocopherol concentration increased an additional 196% (from 27.2 ± 3.2 to 80.4 ± 5.6 μg/g). The hepatic α-tocopherol content of the group LOW (11.9 μg/g) showed that these animals were not vitamin E-deficient.

Sensitivity to in vitro *lipid peroxidation.* No differences in lipid peroxidation were observed between the three vitamin E groups at time 0 (Fig. 1). Incubation in the presence of Fe^{++}-ascorbate significantly increased the production of lipid peroxidation products in the three groups. The rate of increase in lipid peroxidation was significantly higher in group LOW than in the other two groups showing higher liver α-tocopherol levels (Fig. 1). No significant differences between group MEDIUM and group HIGH were found at any incubation time.

We have previously shown (12) that vitamin C supplementation also decreases nonenzymatic *in vitro* lipid peroxidation in the guinea pig liver under the same experimental conditions used here (Fig. 2A). The optimum intermediate level of vitamin C supplementation found in that work, 660 mg of vitamin C/kg of diet, is the one used in the present experiment in all the vitamin E groups. Similar degrees of lipid peroxidation were observed in both works at LOW E plus MEDIUM C (Fig. 2: second bar in A and first bar in B). Although optimum vitamin C levels were used here in the three vitamin E groups, group LOW E showed higher *in vitro* lipid peroxidation values than groups MEDIUM E and HIGH E (Fig. 2B).

Oxidative stress in the hydrophilic compartment. Two indexes of oxidative stress were measured in the hydrophilic compartment, GSH/GSSG ratio, and protein carbonyls. The results obtained for both indexes were highly consistent since no significant changes were observed as a function of vitamin E dietary and hepatic concentration in any group (Table 1).

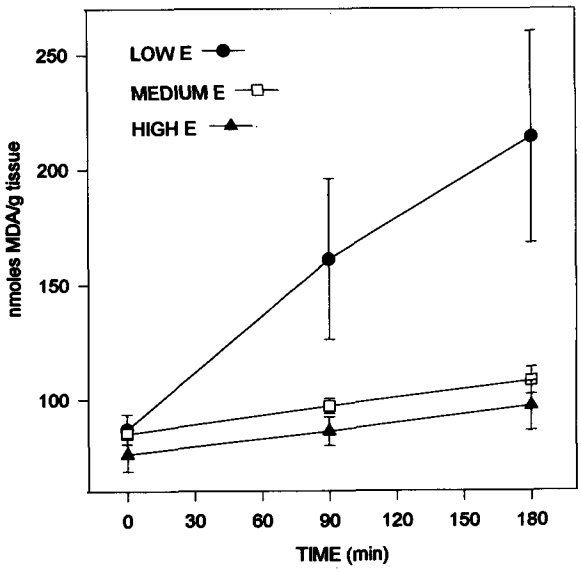


FIG. 1. Sensitivity to *in vitro* nonenzymatic (Fe^{++}-ascorbate) lipid peroxidation in the liver of guinea pigs fed with diets containing 15 (LOW E), 150 (MEDIUM E), or 1,500 (HIGH E) mg of vitamin E/kg during five weeks. Values are means ± SEM and are expressed as nmoles of malondialdehyde (MDA) equivalents/g tissue; n = seven animals per point. *In vitro* lipid peroxidation increased significantly in the three groups as a function of time [one-way analysis of variance (ANOVA); $P < 0.01$ in LOW E; $P < 0.001$ in MEDIUM E and HIGH E]. The rate of increase of liver peroxidation was significantly higher in LOW E than in the other two groups (two-way ANOVA; $P < 0.05$).

Fatty acid analyses. In the phosphatidylethanolamine fraction of liver lipids, significant changes were observed between groups MEDIUM and HIGH. Both unsaturated/saturated ratio (U/S) and the unsaturation index (UI; which takes into account the number of double bonds of each polyunsaturated fatty acid) decreased in group HIGH in relation to group MEDIUM (Table 2 and Fig. 3). This was mainly due to significant decreases in the percentage of α-linolenic (18:3n-3), arachidonic (20:4n-6), n-6 PUFA, and n-3 PUFA and to a significant increase in 16:0. The group LOW only showed a decrease in total n-6 PUFA and no significant changes were detected in any particular fatty acid. PUFA n-6 were maximum in group MEDIUM, and both HIGH and LOW vitamin E levels led to decreases in unsaturated fatty acids.

Phosphatidylcholine showed significant changes of similar character to those observed for phosphatidylethanolamine. The main changes observed occurred again in the group HIGH. n-3 PUFA, U/S ratio, UI, and linolenic acid decreased and saturated 12:0 increased in group HIGH in relation to group MEDIUM (Table 3 and Fig. 3). The unsaturated 20:2 n-6 decreased from group MEDIUM to group LOW.

In the sphingomyelin fraction, the UI, U/S ratio, n-6

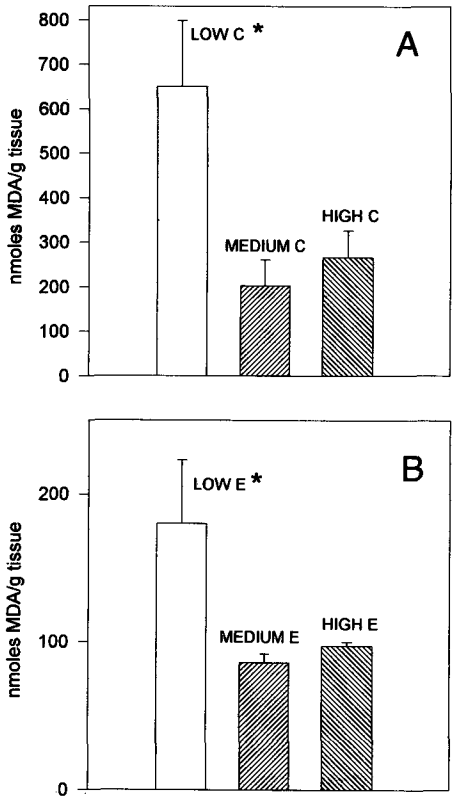


FIG. 2. Comparison of the effects on sensitivity to lipid peroxidation in the liver due to different dietary vitamin C levels in guinea pigs receiving LOW vitamin E (A) with those due to different vitamin E levels in guinea pigs receiving MEDIUM vitamin C in the diet (B). Optimum protection is obtained with the simultaneous presence of intermediate levels of both vitamins (second bar in B). Vitamin E (mg/kg) was: 15 (LOW), 150 (MEDIUM), and 1,500 (HIGH); and vitamin C (mg/kg) was: 33 (LOW), 660 (MEDIUM), and 13,200 (HIGH). Values in (B) come from Barja *et al.* (Ref. 12). Both in A and B the species, strain, body sizes, time of vitamin C or E supplementation, and lipid peroxidation conditions (90 min in the presence of ascorbate-Fe^{++}) were the same. Values are expressed as nmoles of MDA equivalents/g tissue; * = significant difference in relation to the other two groups, $P < 0.05$. See Figure 1 for abbreviation.

TABLE 1
***In Vivo* Oxidative Stress-Related Parameters in the Liver Hydrophylic Compartment of Guinea Pigs Maintained During Five Weeks in Three Diets Differing in Vitamin E Levels**

	mg of Vitamin E/kg diet		
	LOW (15)	MEDIUM (150)	HIGH (1,500)
GSH/GSSG	47.4 ± 7.4	49.1 ± 11.6	39.5 ± 2.2
Protein carbonyls (nmol/mg protein)	4.0 ± 0.9	4.7 ± 1.2	4.0 ± 1.0

[a]Values are means ± SEM from 5–6 (GSH/GSSG), or 7 (protein carbonyls) animals per group.

TABLE 2
Fatty Acid Composition (mol%) of Phosphatidylethanolamine in the Liver of Guinea Pigs Maintained During Five Weeks in Three Diets Differing in Vitamin E Levels[a]

	mg of Vitamin E/kg diet				
	LOW (15)		MEDIUM (150)		HIGH (1,500)
16:0	7.99 ± 0.52		7.64 ± 0.34	*	8.81 ± 1.01
16:1	1.02 ± 0.05		1.03 ± 0.17		1.08 ± 0.26
18:0	36.93 ± 1.59		36.08 ± 1.79		36.99 ± 1.52
18:1	5.93 ± 0.58		5.27 ± 0.58		5.48 ± 0.70
18:2n-6	30.08 ± 2.23		31.04 ± 1.06		32.16 ± 1.72
18:3n-3	2.19 ± 0.81		2.93 ± 0.67	*	1.78 ± 0.86
20:3n-6	0.37 ± 0.03		0.41 ± 0.08		0.44 ± 0.22
20:4n-6	13.05 ± 1.13		13.35 ± 1.20	*	11.39 ± 2.13
22:5n-3	1.05 ± 0.12		0.99 ± 0.14		0.95 ± 0.14
22:6n-3	0.88 ± 0.10		0.93 ± 0.18		0.89 ± 0.16
SFA	44.92 ± 1.32		43.73 ± 1.55		45.80 ± 2.31
PUFA n-6	45.70 ± 1.70	*	47.74 ± 1.61	*	45.78 ± 1.63
PUFA n-3	4.13 ± 0.91		4.85 ± 0.61	*	3.63 ± 0.92
MUFA	6.95 ± 0.60		6.31 ± 0.55		6.56 ± 0.83
U/S	1.21 ± 0.06		1.28 ± 0.08	*	1.18 ± 0.09
UI	3.06 ± 0.19		3.26 ± 0.24	*	2.92 ± 0.30
Chain length	18.06 ± 0.16		18.12 ± 0.10		18.06 ± 0.16

[a]Values are means ± SEM from seven animals per group. U/S is the ratio between Σmol% of unsaturated and Σmol% saturated fatty acids. The unsaturation index (UI) corresponds to the ratio: (Σmol% of unsaturated fatty acids × number of double bonds of each unsaturated fatty acid)/Σmol% of saturated fatty acids (SFA), PUFA n-6: Polyunsaturated fatty acids n-6; (Σmol% 18:2 + 20:3 + 20:4 + 22:4). PUFA n-3: Polyunsaturated fatty acids n-3; (Σmol% 18:3 + 22:5 + 22:6). MUFA: Monounsaturated fatty acids (Σmol% 16:1 + 18:1). Average chain length = $[(\Sigma\%Total_{14} \times 14) + (\Sigma\%Total_{16} \times 16) + (\Sigma\%Total_{18} \times 18) + (\Sigma\%Total_{20} \times 20) + (\Sigma\%Total_{22} \times 22)]/100$. Asterisks between columns describe differences between LOW E and MEDIUM E or between MEDIUM E and HIGH E. * = $P < 0.05$.

PUFA, arachidonic and linoleic acid decreased in group HIGH in relation to group MEDIUM (Table 4 and Fig. 3). Even though 16:0 was decreased in group HIGH in relation to group MEDIUM, this was more than compensated by an increase in 18:0, and saturated fatty acids (SFA) did not differ between the two groups. The only changes observed between groups MEDIUM and LOW were a decrease in n-6 PUFA and a decrease in the U/S ratio. Thus, again both low and high vitamin E levels caused a reduced percentage of unsaturated fatty acids, and the changes were more marked in HIGH than in LOW (but nondeficient) vitamin E animals.

More significant and quantitatively greater changes were observed for lysophosphatidylcholine, in which alterations followed the same pattern as in the rest of membrane phospholipid fractions. Group HIGH showed very significant decreases in n-6 PUFA, U/S ratio, UI, and arachidonic acid and important increases in 18:0 in relation to group MEDIUM (Table 5). The increase in 18:0, similarly to what was observed for sphingomyelin, compensated for the decrease in 16:0 and in this case the increase in the percentage of SFA in group HIGH reached statistical significance. Decrease in vitamin E from MEDIUM to LOW groups resulted in highly significant decreases in UI, n-6 PUFA, and arachidonic acid, and in increased 18:0, decreased 16:0, and increased total SFA. When both increases or decreases were considered, the changes observed with HIGH and LOW vitamin E levels in relation to group MEDIUM pointed always in the same direc-

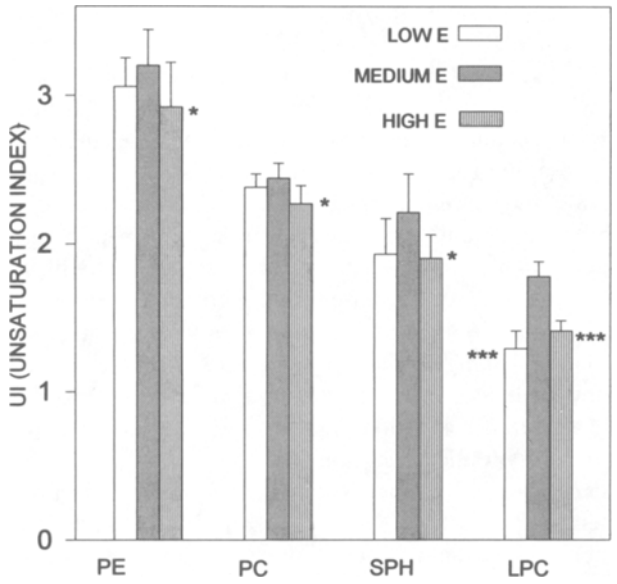


FIG. 3. Decreases in fatty acid unsaturation with both LOW and HIGH vitamin E dietary supplementation in liver guinea pig phospholipids. Vitamin E (mg/kg) was: 15 (LOW), 150 (MEDIUM), and 1,500 (HIGH). PC = phosphatidylcholine; PE = phosphatidylethanolamine; SPH = sphingomyelin; LPC = lysophosphatidylcholine; UI = unsaturation Index (see Tables 2–5). Asterisks: significant differences respect to group MEDIUM (* = $P < 0.05$; *** = $P < 0.001$).

TABLE 3
Fatty Acid Composition (mol%) of Phosphatidylcholine in the Liver of Guinea Pigs Maintained During Five Weeks in Three Diets Differing in Vitamin E Levels[a]

	mg of Vitamin E/kg diet		
	LOW (15)	MEDIUM (150)	HIGH (1,500)
12:0	0.06 ± 0.01	0.05 ± 0.01 *	0.07 ± 0.01
14:0	0.16 ± 0.02	0.14 ± 0.02	0.18 ± 0.04
16:0	15.80 ± 0.56	15.39 ± 0.87	16.44 ± 1.42
16:1	1.15 ± 0.32	0.83 ± 0.23	0.95 ± 0.26
18:0	28.44 ± 0.97	28.79 ± 0.39	29.24 ± 2.03
18:1	12.44 ± 1.11	12.91 ± 1.42	12.02 ± 0.69
18:2n-6	35.51 ± 0.59	34.98 ± 1.80	35.16 ± 1.25
18:3n-3	1.08 ± 0.44	2.00 ± 1.15 *	0.87 ± 0.13
20:2n-6	0.43 ± 0.03 *	0.49 ± 0.04	0.47 ± 0.08
20:3n-6	0.77 ± 0.13	0.80 ± 0.20	0.78 ± 0.19
20:4n-6	2.82 ± 0.40	2.97 ± 0.32	2.85 ± 0.45
22:4n-6	0.28 ± 0.03	0.30 ± 0.05	0.34 ± 0.03**
22:5n-3	0.15 ± 0.03	0.17 ± 0.02	0.16 ± 0.01
22:6n-3	0.25 ± 0.05	0.22 ± 0.03	0.21 ± 0.02
SFA	44.47 ± 1.31	44.39 ± 0.87 *	45.94 ± 1.40
PUFA n-6	40.48 ± 0.41	41.07 ± 1.54	40.03 ± 1.38
PUFA n-3	1.49 ± 0.42	2.40 ± 1.13 *	1.25 ± 0.11
MUFA	13.59 ± 1.26	13.75 ± 1.60	12.97 ± 0.81
U/S	1.23 ± 0.06	1.25 ± 0.04 *	1.17 ± 0.06
UI	2.38 ± 0.09	2.44 ± 0.10 *	2.27 ± 0.12
Chain length	17.65 ± 0.06	17.79 ± 0.07	17.71 ± 0.10

[a]Values are means ± SEM from seven animals per group. Asterisks at right of HIGH E values describe differences between HIGH E and LOW E. *$P < 0.05$; **$P < 0.01$. For other explanations and abbreviations see Table 2.

TABLE 4
Fatty Acid Composition (mol%) of Sphingomyelin in the Liver of Guinea Pigs Maintained During Five Weeks in Three Diets Differing in Vitamin E Levels[a]

	mg of Vitamin E/kg diet		
	LOW (15)	MEDIUM (150)	HIGH (1,500)
16:0	12.13 ± 2.71	10.43 ± 1.62 *	8.42 ± 0.97**
16:1	0.73 ± 0.09	0.79 ± 0.10	0.78 ± 0.17
18:0	38.97 ± 4.46	37.96 ± 3.62 *	42.66 ± 3.30
18:1	9.80 ± 1.52	10.29 ± 1.42	10.94 ± 2.18
18:2n-6	21.79 ± 2.85	24.43 ± 4.09 *	21.14 ± 1.34
18:3n-3	2.34 ± 0.50	2.76 ± 0.80	2.55 ± 0.79
20:3n-6	1.79 ± 0.50	1.82 ± 0.68	2.31 ± 0.48
20:4n-6	8.00 ± 1.39	8.74 ± 1.48 *	7.29 ± 1.04
22:0	2.25 ± 0.64	2.36 ± 0.61	2.62 ± 0.38
22:6n-3	1.03 ± 0.42	1.00 ± 0.21	1.10 ± 0.33
SFA	53.37 ± 3.73	50.76 ± 2.62	53.71 ± 3.04
PUFA n-6	33.93 ± 2.51 *	37.76 ± 3.72 *	33.31 ± 1.41
PUFA n-3	2.82 ± 0.70	2.83 ± 0.76	3.41 ± 0.48
MUFA	10.53 ± 1.47	11.08 ± 1.39	11.73 ± 2.15
U/S	0.85 ± 0.11 *	0.98 ± 0.10 *	0.86 ± 0.09
UI	1.93 ± 0.24	2.21 ± 0.26 *	1.90 ± 0.16
Chain length	17.86 ± 0.31	18.23 ± 0.14	18.13 ± 0.19

[a]Values are means ± SEM from seven animals per group. * = $P < 0.05$; ** = $P < 0.01$. For other explanations and abbreviations see Table 2.

TABLE 5
Fatty Acid Composition (mol%) of Lysophosphatidylcholine in the Liver of Guinea Pigs Maintained During Five Weeks in Three Diets Differing in Vitamin E Levels[a]

	mg of Vitamin E/kg diet		
	LOW (15)	MEDIUM (150)	HIGH (1,500)
16:0	8.80 ± 1.13 *	11.88 ± 1.87 ***	7.94 ± 0.76
18:0	53.46 ± 3.04 ***	44.96 ± 2.95 ***	52.52 ± 1.34
18:1	9.23 ± 0.83	8.92 ± 1.16	9.69 ± 0.91
18:2n-6	21.48 ± 1.95	22.42 ± 1.63	22.55 ± 1.44
20:4n-6	7.07 ± 0.85 ***	11.91 ± 1.09 ***	7.65 ± 0.35
SFA	62.27 ± 1.94 ***	56.85 ± 2.00 *	60.47 ± 1.83**
PUFA n-6	28.56 ± 2.47 ***	34.33 ± 1.85 ***	30.20 ± 1.46
MUFA	9.23 ± 0.83	8.92 ± 1.16	9.69 ± 0.91
U/S	0.60 ± 0.04	0.76 ± 0.04 ***	0.66 ± 0.04*
UI	1.29 ± 0.12 ***	1.78 ± 0.10 ***	1.41 ± 0.07*
Chain length	17.97 ± 0.03	18.01 ± 0.23	18.06 ± 0.23

[a]Values are means ± SEM from seven animals per group. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. For other explanations and abbreviations see Table 2.

tion: decreases in the degree of total fatty acid unsaturation (Fig. 3).

The changes observed in the triglyceride fraction were totally different from those observed for phospholipids mainly derived from membrane fractions. Group HIGH showed increases in 14:0 and 16:0 and a decrease in 18:0 in relation to group MEDIUM, and an increase in the percentage of SFA in relation to group LOW (Table 6). Group LOW showed higher 18:2 n-6 and n-6 PUFA and lower 18:0 than group MEDIUM. Neither the U/S ratio nor the UI showed changes as a function of vitamin E levels (Table 6).

TABLE 6
Fatty Acid Composition (mol%) of Triglycerides in the Liver of Guinea Pigs Maintained During Five Weeks in Three Diets Differing in Vitamin E Levels[a]

	mg of Vitamin E/kg diet		
	LOW (15)	MEDIUM (150)	HIGH (1,500)
12:0	0.83 ± 0.20	0.94 ± 0.04	0.88 ± 0.13
14:0	0.34 ± 0.06	0.35 ± 0.04 **	0.43 ± 0.04
16:0	27.68 ± 2.80	26.31 ± 1.75 *	30.98 ± 4.45*
16:1	0.80 ± 0.17	0.95 ± 0.06	0.88 ± 0.10
18:0	8.95 ± 0.44 ***	11.79 ± 1.23 **	9.27 ± 1.53
18:1	22.14 ± 0.46	22.14 ± 1.44	21.47 ± 1.76
18:2n-6	30.78 ± 0.90 ***	28.91 ± 0.31	29.20 ± 3.40
18:3n-3	4.72 ± 0.77	4.85 ± 0.80	5.20 ± 0.25
20:4n-6	1.46 ± 0.30	1.54 ± 0.11	1.53 ± 0.12
22:6n-3	0.73 ± 0.17	0.68 ± 0.04	0.61 ± 0.09
SAF	37.81 ± 2.42	39.40 ± 2.93	41.57 ± 3.09*
PUFA n-6	36.96 ± 1.07 *	35.30 ± 1.12	35.94 ± 3.65
PUFA n-3	5.46 ± 0.86	5.53 ± 0.77	5.81 ± 0.31
MUFA	22.94 ± 0.64	23.10 ± 1.40	22.35 ± 1.87
U/S	1.61 ± 0.12	1.50 ± 0.15	1.43 ± 0.22
UI	2.89 ± 0.23	2.70 ± 0.27	2.58 ± 0.41
Chain length	15.53 ± 0.38	15.04 ± 0.24	15.76 ± 0.16

[a]Values are means ± SEM from seven animals per group. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. For other explanations and abbreviations see Table 2.

The mean chain length was not modified in any lipid fraction as a result of vitamin E treatment.

DISCUSSION

In the present study, we have used three different levels of vitamin E in the diet to obtain guinea pigs with three different hepatic concentrations of vitamin E. Animals in the LOW vitamin E group ingested 0.6–0.75 mg of vitamin E per day, a dose very close to the minimum daily vitamin E requirement for long-term maintenance of the growing guinea pig, 1 mg/d (18,19). Animals in this group LOW have low tissue vitamin E levels, but they cannot be considered vitamin E-deficient. Animals in group MEDIUM are in the normal range used for routine maintenance of guinea pig, receiving an amount of vitamin E more than sixfold higher than the minimum daily requirement. Finally, group HIGH was designed to clarify the effects of supplementing the diet with levels of vitamin E 65-fold higher than the minimum daily requirement on hydrophilic and lipid oxidative stress markers and fatty acid profiles in the guinea pig liver.

In spite of the presence of widely different hepatic α-tocopherol contents, neither GSH/GSSG ratio or protein carbonyls varied. The reduced and oxidized forms of glutathione are present in the hydrophilic compartment, and the same is true for a great part of tissue proteins. That result is consistent with the function of vitamin E as an important antioxidant in membranes but not in the hydrophilic cellular compartment.

Contrasting with this lack of effect in the hydrophilic compartment, the increase in liver α-tocopherol from group LOW to group MEDIUM strongly inhibited nonenzymatic (induced by Fe^{++}-ascorbate) *in vitro* lipid peroxidation. This is consistent with an important antioxidant role of vitamin E in the membrane cellular compartment. Further increases in liver α-tocopherol to group HIGH did not afford additional protection in our system, perhaps because under these conditions ascorbate eventually becomes limiting. Some reports have shown similar results for laboratory rodents. A diet with 180 mg of vitamin E/kg decreased lipid peroxidation in relation to a diet with 86 mg/kg in rat liver after almost one month, whereas 1,400 mg/kg did not bring further protection (20). Vitamin E dietary supplementation also decreased susceptibility to lipid peroxidation in rat liver (21,22), and peroxidation of rat liver microsomes induced by Fe^{++}-ascorbate (23). No decreases in liver peroxidation were described in mice treated for 7 wk with 200 mg of vitamin E/kg vs. a deficient diet, but tissue vitamin E levels were not measured (24). The reason for the lack of differences in *in vitro* lipid peroxidation between groups MEDIUM and HIGH is not known, but the possibility exists that most ascorbate is consumed during the first part of prolonged incubation. It is possible that the amount of oxidative stress generated by our incubation system was not high enough to produce a vitamin E depletion which could discriminate between MEDIUM and HIGH vitamin E levels.

Previous work from our laboratory showed that vitamin C supplementation decreases nonenzymatic *in vitro* lipid peroxidation in LOW E guinea pig liver under the same experimental conditions used here. In the present work, we show that vitamin E protects from *in vitro* lipid peroxidation in spite of the use—in the three vitamin E groups—of the optimum (intermediate) vitamin C concentration found in our earlier work (12). Thus, optimum protection from hepatic *in vitro* lipid peroxidation in the guinea pig needs the simultaneous supplementation with both vitamins (C and E) at intermediate levels. This is consistent with the proposal of a cooperation between both vitamins *in vivo*, vitamin C reducing α-tocopheroxyl radical back to vitamin E (25,26).

Unsaturated fatty acids are highly sensitive molecules to free radical damage in cells. This sensitivity increases as a function of the number of double bonds of each individual fatty acid (27). Few works have addressed the problem of the possible effects of dietary vitamin E on tissue fatty acid profiles (28), as we have here in liver, and to our knowledge none has been performed in guinea pigs. A decrease in fatty acid unsaturation in liver microsomes of rat pups fed a vitamin E-deficient diet during 9 mon, the mothers receiving the same diet during gestation, has been recently described (29). In this work, liver vitamin E decreased to levels much lower than in our work (0.8 μg/g liver) and decreases in unsaturated 16:1, 18:1, 18:2, 20:3, and increases in 18:0 and total SFA were found in the vitamin E-deficient group. No group supplemented with vitamin E megadoses was included in that study. Other authors also have found decreases in unsaturation in liver microsomes and mitochondria in vitamin E-deficient rats

(30). At the other end of the spectrum, 2,000 mg of dietary vitamin E/kg diet during 6 wk decreased UI, 20:4, and 22:6 and increased 18:2 in the liver of spontaneously hypertensive rats (31). No group with low E levels was included in this work, only phosphatidylcholine was studied, and SFA were not measured. Other authors did not find changes in fatty acid unsaturation in rat tissues after feeding low vitamin E diets (32). Problems in study design may explain differences in results reported. As an example, no changes in liver PUFA were found after feeding Swiss mice diets with 0, 50, or 150 mg of vitamin E/kg, but no changes in liver vitamin E were found among the three dietary groups (33). Clearly, the lack of effect on PUFA in this study was associated with a lack of change in liver vitamin E.

In our present work, we have shown that both LOW and HIGH dietary and hepatic vitamin E levels were associated with a significantly lower fatty acids percentage of unsaturated, and maximum unsaturation occurs at intermediate vitamin E levels (Fig. 3). The LOW E effect is observed even in animals in which tissue vitamin E concentrations have not fallen to deficiency levels. Quantitatively greater effects were observed at HIGH than at LOW dietary vitamin E. When significant changes were observed, they occurred in the same direction in all the lipid fractions except for the triglycerides, indicating that the protective antioxidant role of vitamin E at intermediate levels operates mainly in membrane lipids. Triglycerides are deposited in hepatic cells surrounded by other molecules like phospholipids or cholesterol. This may afford them protection against oxidative stress, thus explaining their minor changes after vitamin E supplementation in comparison with those shown by phospholipids. The changes observed in phospholipids always occurred in the same direction from the group MEDIUM to the other two groups: decreases in the percentage of unsaturated fatty acids.

The decrease in unsaturation at low vitamin E levels could be predicted from prior work on the antioxidant role of this vitamin in the lipid membrane environment. Concerning the relative reduction of unsaturation at high vitamin E doses, various possibilities arise. It has been shown that α-tocopherol can have a prooxidant effect in chemical (10) and low density lipoprotein systems (11) *in vitro*. The prooxidant effect has been attributed to the reaction of tocopheroxyl radical (the oxidized free-radical form of vitamin E) with unsaturated lipids and lipid hydroperoxides, forming alkyl and peroxyl lipid radicals (10). This prooxidant effect does not occur if sufficiently high concentrations of antioxidants like vitamin C or ubiquinol-10, which reduce tocopheroxyl radical to vitamin E, are present in the *in vitro* system (11). This explanation would be consistent with our *in vitro* results, since the dietary vitamin E level of the group HIGH (1,500 mg/kg) surpassed that of vitamin C (660 mg/kg) in the present work. Nevertheless, no increases in oxidative stress markers (GSH/GSSG and protein carbonyls) were found in group HIGH. Furthermore, only percent, not absolute, fatty acid values were measured. Thus, the decreases in percent unsaturated fatty acids can be due to increases in SFA instead of to decreases in absolute amounts of unsaturates. Other alternatives are also possible. Effects of vitamin E on the synthesis of unsaturated fatty acids cannot be discarded since it has been shown that Δ-9 desaturase activity is decreased both by vitamin E-deficient and by vitamin E- supplemented diets in rat liver microsomes (34) and that high vitamin E concentrations (20-fold the control level) decrease Δ-6 desaturase in rat liver (35). Finally, the decrease in unsaturation in group HIGH also can be due to an inhibitory effect of high doses of α-tocopherol on phospholipase activity (36). This would decrease the elimination from the membranes of lipid hydroperoxides, known propagators of lipid peroxidation. In any case, the results show decreases in the percentage of unsaturated membrane fatty acids at both too low and too high dietary vitamin E doses in the liver *in vivo*.

A decrease in liver fatty acid unsaturation with both too low (33 mg/kg) and too high (13,200 mg/kg) dietary vitamin C levels has been found in our laboratory in the same guinea pig model used here, and higher unsaturation was observed again at intermediate (660 mg/kg) vitamin C dietary levels (12). These intermediate ascorbate levels were also enough to protect against lipid peroxidation. For both antioxidant vitamins (E and C), an intermediate dietary vitamin level exists at which protection from lipid peroxidation and a percentage of unsaturated higher fatty acids is observed in the liver. That level is 40- (vitamin C) or 6-fold (vitamin E) higher than the minimum daily requirement needed to avoid deficiency syndromes. This is consistent with the idea that optimum dietary levels of vitamin E and C are probably higher than the current RDA, whereas megadose levels can alter membrane fatty acid profiles in the same direction as marginal antioxidant vitamin deficiency.

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Fatty Acid Composition of Danish Margarines and Shortenings, with Special Emphasis on *trans* Fatty Acids

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ABSTRACT: *Trans* fatty acids from hydrogenated vegetable and marine oils could be as hypercholesterolemic and atherogenic as saturated fatty acids. Hence, it is important to know the fatty acid composition in major food contributors, e.g., margarines and shortenings. In 1992 margarines were examined, and in 1995 brands covering the entire Danish market were examined. Significant amounts of *trans*-18:1 were found only in hard margarines (mean: 4.2 ± 2.8%) and shortenings (mean: 6.8 ± 3.1%), whereas the semisoft and soft margarines contained substantially less *trans*-18:1 in 1995 than in 1992. Where marine oils had been used to a larger degree the mean *trans*-monoenoic content was about 15%, of which close to 50% was made up of long-chain (C_{20} and C_{22}) *trans* fatty acids. A noteworthy decrease in the content of *trans*-18:1 had occurred for the semisoft margarines, from 9.8 ± 6.1% in 1992 to 1.2 ± 2.2% in 1995. Calculated from sales figures, the supply of *trans*-18:1 plus saturated fatty acids from margarines has decreased over this three-year period by 1.4 g/day, which has been replaced by *cis* monounsaturated and polyunsaturated fatty acids.

Lipids 31, 971–975 (1996).

Trans fatty acids (TFA) are formed in small amounts in ruminant animals and will appear in the meat and milk (1). Consequently, TFA have always been part of the human diet. However, an additional and a more recent intake is derived from industrially hydrogenated vegetable and marine oils used in the manufacturing of margarines and shortenings, where mainly *trans*-octadecanoic acid is found. The position of the double bond is more or less normally distributed from the n-2 to the n-12 position with n-7, n-8, and n-9 being the most abundant (2).

Recently, the contribution of dietary TFA to plasma lipid and lipoprotein profiles (3–10) and risk of coronary heart disease (11–13) has attracted much scientific interest. Although experts have differed somewhat in their interpretation of the scientific evidence, and how the evidence should influence dietary advice to the public (14–16), many would admit that products high in TFA are unnecessary and could be harmful.

Thus, in Denmark the margarine industry has been put under pressure to manufacture products with lesser contents of TFA. Furthermore, it might be feared that TFA in margarines have been replaced by atherogenic saturated fatty acids, primarily lauric (LA; C_{12}), myristic (MY; C_{14}), and palmitic acid (PA; C_{16}) (6,17,18). From a public health perspective this would be an unfortunate development. To get more insight into the changing fatty acid pattern in Danish margarine products, we decided to examine the fatty acid composition of available margarines in 1995, and compare it to a similar examination performed in 1992.

EXPERIMENTAL PROCEDURES

All Danish margarine brands available on the retail market in the autumn of 1992 and 1995 were analyzed. Furthermore, in 1995 all Danish manufactured shortening brands intended for use in the baking industry were analyzed (in Denmark, shortenings are sold only for industrial purposes). One package of 400–500 g of each brand was taken at random directly from the production line.

In 1992 three factories produced margarine in Denmark, namely Vejle Margarinefabrik (Vejle, Denmark), Dragsbæk Margarinefabrik (Thisted, Denmark), and Van den Bergh Foods (Sønderborg, Denmark). In 1995 one more factory, Grønvang Margarinefabrik (Vejen, Denmark), was in existence. From 1992 to 1995 large changes in product types and brand names of margarines had occurred.

There was only minimal import of margarine and shortening in 1992 and 1995, so the sampling covers the entire Danish market for the retail outlet and the baking industry.

A total of 117 samples was drawn consisting of 74 brands of margarines (40 margarines in 1992 and 34 margarines in 1995) for the retail market, and 43 brands of shortenings for the baking industry.

The fatty acids were determined by boiling the fat in methanolic potassium hydroxide, methylation by boiling with methanol and boron trifluoride, and extraction of the fatty acid methyl esters with isooctane, followed by gas–liquid

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Abbreviations: GLC, gas–liquid chromatography; HDL, high density lipoprotein; LA, lauric acid; LC, long-chain fatty acids (C_{20} or longer; saturated and unsaturated); LDL, low density lipoprotein; LI, linoleic acid; MY, myristic acid; PA, palmitic acid; TFA, *trans* fatty acids.

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chromatography (GLC) (PE 8500; Perkin Elmer Corp., Norwalk, CT) on a 50-m, capillary column, CP Sil 88, 0.32-mm i.d. (Chrompack International, Middelburg, The Netherlands) with flame-ionization detection. C_{17} is used as an internal standard for quantification of the fatty acids (19). Temperature program was: start temperature, 120°C; iso time, 0.0; ramp rate, 2.0°C; oven temperature, 220°C; iso time, 2.0 min; detector temperature, 350°C; programmed temperature, vaporizer injection system from 40 to 300°C. With this temperature program and column, there is good separation between *cis*- and *trans*-18:1, but there is still a minor overlap between some of the isomers, tending to underestimate the *trans* content a little. However, the overlap is negligible for *trans*-18:1 amounts below 10% of the fatty acids (20). *Cis*-20:1 elutes before 18:3, so the *trans*-20:1 does not interfere with 18:3.

All analyses were carried out as double determinations, and the results were ascertained by making the so-called R-charts over the deviation on the double determination of the fatty acids PA (16:0), stearic (18:0), oleic (*cis*-18:1n-9), and linoleic (LI; *cis*-18:2n-6). As reference materials are used and in-house material of margarine, certified BCR reference material CRM 162 Soya-maize oil (Community Bureau of Reference, Brussels, Belgium) and Nu-Chek-Prep standards GLC 68 and 17A[1] (Nu-Chek-Prep, Inc., Elysian, MN).

The long-chain (LC) TFA from hydrogenated fish oils cannot be sufficiently separated to be quantified by GLC on a 50-m polar capillary column. To quantify these TFA, the fatty acid methyl esters were separated by thin-layer chromatography by application in a spot on Kieselgel plates (Merck, Darmstadt, Germany) impregnated with a solution of 5% silver nitrate and eluted with petroleumether/diethyl ether. The spots were visualized by spraying with dichlorfluorescin, scraped off and eluted with diethyl ether (21). The spots with saturated fatty acids and TFA were combined. The fatty acids were then determined as described previously, again using C_{17} from the spot with saturated fatty acids as internal standard for the determination of TFA.

Statistical analysis was performed with the Student's unpaired *t*-test. Two-sided *P*-values less than 0.05 were considered statistically significant. Values are given as mean ±1 SD unless otherwise indicated.

RESULTS

For presentation, the margarines for the retail market are divided into three categories based on their LI content: (i) margarines with less than 20% (wt% of total fatty acids) LI (these are hard margarines used mainly for frying); (ii) margarines containing 20–40% LI (these are semisoft margarines used mainly as spreads on bread); (iii) margarines with more than 40% LI. These are soft margarines also used as spreads.

The contents (mean, range) of individual fatty acids in margarines and shortenings are given in Table 1. *Trans*-18:2 or conjugated 18:2 are not present in Danish margarines in any significant amounts (<0.2% of total fatty acids).

A nonsignificant fall in *trans*-18:1 from 1992 to 1995 in hard margarines, from 5.8 ± 3.2 to 4.2 ± 2.8% ($P = 0.2$), was demonstrated. Three hard margarine products (15%) were "*trans*-free" (containing less than 1%) in 1995 as opposed to none in 1992. Total saturated fatty acids were higher in 1992 than in 1995 (41.9 ± 2.8 and 34.6 ± 11.2%, respectively), even though not significantly ($P = 0.07$). The saturated fatty acids LA, MY, and PA, suspected to have a high atherogenic potential, were almost unchanged in 1992 compared to 1995 (29.4 ± 4.0 and 26.5 ± 8.5%, respectively). Total monounsaturated fatty acids—chiefly oleic acid—were 29.9 ± 7.5% in 1992, significantly lower than

TABLE 1
Fatty Acid Composition (wt% of total, mean, range) of Danish margarines in 1992 and 1995, and Shortenings (in 1995)

	Margarines						Shortenings (<20% linoleic acid)
	<20% Linoleic acid		(20–40% linoleic acid)		>40% Linoleic acid		
	1992	1995	1992	1995	1992	1995	1995
Fatty acid	(*n* = 9)	(*n* = 20)	(*n* = 17)	(*n* = 8)	(*n* = 14)	(*n* = 6)	(*n* = 43)
C_8	—[a]	0.4; 0–2.1	0.3; 0–3.8	0.5; 0–1.6	—	0.6; 0–2.0	0.1; 0–2.2
C_{10}	—	0.3; 0–1.4	0.2; 0–2.7	0.4; 0–1.1	—	0.4; 0–1.3	0.1; 0–1.5
C_{12}	0.5; 0–1.7	2.3; 0–8.8	2.0; 0–21.4	4.5; 0–11.1	0.8; 0–2.9	3.9; 0–8.7	1.1; 0–9.9
C_{14}	3.6; 1.7–6.2	2.3; 0–8.4	1.0; 0–7.6	1.8; 0–3.6	0.4; 0–1.1	1.6; 0–3.5	2.0; 0–7.0
C_{16}	25.4; 20.5–34.2	22.0; 10.5–33.7	17.0; 7.7–23.4	19.1; 5.6–22.0	12.5; 8.8–19.4	14.0; 11.7–17.8	25.8; 19.2–37.0
16:1	0.4; 0–1.9	0.2; 0–1.7	0.1; 0–2.2	—	—	—	0.2; 0–1.5
C_{18}	9.0; 5.8–16.5	6.4; 2.9–9.2	5.9; 3.7–11.2	7.6; 3.9–20.8	6.7; 5.7–8.5	6.6; 4.2–10.7	6.7; 3.3–15.1
Trans-18:1	5.8; 1.4–10.6	4.2; 0–8.2	9.8; 0–22.3	1.2; 0–5.8	1.9; 0–9.6	1.3; 0–5.6	6.8; 0–13.7
Cis-18:1	28.5; 13.6–37.4	39.3; 17.9–52.9	32.7; 13.3–42.2	32.4; 14.8–46.0	20.5; 17.0–23.3	21.1; 15.3–29.0	37.4; 17.4–48.0
18:2	12.1; 7.3–16.9	14.0; 4.2–19.8	25.4; 11.3–38.9	31.5; 20.5–40.0	55.9; 41.2–61.1	49.2; 41.1–53.8	11.9; 6.3–18.7
18:3	2.9; 1.2–4.0	4.4; 1.7–7.3	3.1; 0–6.0	4.1; 0.9–6.4	0.1; 0–0.6	0.8; 0–1.4	3.5; 1.3–5.3
C_{20}	1.8; 0–2.9	0.8; 0–4.0	0.2; 0–0.9	0.3; 0–1.0	—	—	0.7; 0–3.8
20:1	1.1; 0–2.2	0.8; 0–2.2	0.3; 0–2.5	0.4; 0–1.0	—	—	0.6; 0–2.0
C_{22}	1.7; 0–2.8	0.5; 0–1.8	0.1; 0–0.7	0.4; 0–2.3	0.6; 0–1.1	0.3; 0–1.0	0.5; 0–3.8
22:1	—	—	—	—	—	—	—
Other[b]	7.6; 1.3–15.8	2.2; 0–19.2	1.8; 0.3–19.2	—	0.7; 0–0.9	—	5.4; 0–17.2

[a]Not detected or trace amounts (<0.1%).
[b]Include mainly isomers of long-chain fatty acids.

TABLE 2
***Trans* Monoenoic Fatty Acids (wt% of total fatty acids) in Danish Margarines and Shortenings**

	Mean ± SD	Variation
Trans-16:1	2.4 ± 1.4%	0–5.4%
Trans-18:1	5.7 ± 1.9%	2.6–10.0%
Trans-20:1	4.0 ± 2.2%	0.1–7.6%
Trans-22:1	2.9 ± 2.1%	0–6.1%
Σ*Trans*-monoenoic	15.1 ± 4.9%	8.2–22.2%

40.1 ± 10.8% in 1995 ($P < 0.05$). LC were present in amounts of less than 1% of total fatty acids in none of the brands in 1992, compared to seven brands (35%) in 1995.

In margarines with 20–40% LA, a significant fall in *trans*-18:1 was demonstrated between 1992 and 1995. Mean values were 9.8 ± 6.1% in 1992 and 1.2 ± 2.2% in 1995 ($P < 0.001$). Six brands (75%) contained less than 1% *trans*-18:1 in 1995 compared to only one margarine brand (6%) in 1992. Saturated fatty acids showed no changes (20.1 ± 5.2 and 21.3 ± 6.9%, respectively), and polyunsaturates were significantly higher in 1995 compared to 1992 (28.6 ± 5.9 and 35.8 ± 7.3%, respectively; $P < 0.05$). LC, usually in small amounts, were demonstrated in seven products (41%) in 1992 and in three products (38%) in 1992.

Trans-18:1 was very low in soft margarines in 1992, as well as in 1995. All but four margarine brands (71%) were "*trans*-free" in 1992, and all but two (67%) in 1995. Total saturated fatty acids were significantly higher in 1995 compared to 1992 (21.0 ± 2.3 and 27.4 ± 8.0%, respectively; $P < 0.05$), due entirely to a higher content of LA, MY, and PA (13.6 ± 2.4 and 19.6 ± 4.3%, respectively; $P < 0.001$). LC were not used to any significant degree in the soft, and semisoft, margarines.

The content of saturated LA, MY, and PA was not significantly different ($P = 0.3$) in "*trans*-free" margarines (27.8 ± 11.9%) compared to margarines with >1% *trans*-18:1 (24.2 ± 7.7%).

All shortenings for the baking industry contain less than 20% LI. A lower *trans*-18:1 content was demonstrated in the hard margarines (1995) compared to the shortenings. Mean values in shortenings were 6.8 ± 3.1%, significantly higher than in margarines ($P < 0.005$). Only three (7%) brands of shortenings were "*trans*-free." The higher *trans* content was mainly compensated for by a lower content of polyunsaturated fatty acids.

A total of 13 shortenings and hard margarines contained more than 5% LC. These brands had an average of about 15% total *trans*-monoenoic fatty acids, of which close to 50% were made up of *trans*-LC (20:1 and 22:1) (Table 2). n-3 Polyunsaturated fatty acids are not present in significant amounts (<0.2% of total fatty acids) after hardening of the fish oils.

DISCUSSION

Discussions about the health effects of TFA were intensified in 1990 when TFA were reported to increase low density lipoprotein-(LDL) and decrease high density lipoprotein-(HDL) cholesterol concentrations in the blood of healthy normocholesterolemic subjects (5). Later studies indicated that TFA might also increase plasma triglyceride (4) and lipoprotein(a) concentrations (3,7), both considered to be risk factors for coronary heart disease. The significance of these findings was supported by recent prospective epidemiological studies showing association between the intake of TFA and subsequent coronary heart disease (11–13). Recently, however, several reviews have critically examined the scientific evidence linking the intake of TFA to premature atherosclerosis (14,15,22,23). The conclusions from these reviews are that TFA increase plasma concentrations of total- and LDL-cholesterol when substituted for oleic acid, but decrease cholesterol concentrations when substituted for saturated fatty acids (butter or PA), and that the effects on HDL-cholesterol and lipoprotein(a) concentrations are ambiguous. Also, in some epidemiological studies, the association between intake of TFA and coronary heart disease could not be demonstrated (24,25). Studies on the implications for human health of TFA are still quite few, especially if compared to the wealth of data linking intake of saturated fatty acids to the development of coronary heart disease.

Although data supporting the relation between the intake of TFA and coronary heart disease are sparse, there has, for the last five years, been a public pressure on margarine producers to reduce TFA in their products. In Denmark, the pressure during recent years has been increased further due to the recommendations from the Danish Nutrition Council to reduce the content of TFA in all margarines (16), and the contemplations of the Danish National Food Agency concerning legislation on maximum content of TFA in margarines and other hydrogenated oils (26).

Three saturated fatty acids in the diet—LA, MY, and PA—are involved in raising cholesterol levels as much or more than *trans*-18:1 (6,17,18), whereas the saturated stearic acid almost certainly is biologically neutral (27). From a public health aspect, it would not be appropriate to substitute the *trans* content in margarines with vegetable fats with high contents of LA, MY, and PA, e.g., coconut or palm oil.

This does not seem to be the case for Danish margarines. In the margarines with the most noteworthy reduction in *trans*-18:1 content (from 9.8 to 1.2%), i.e., the semisoft spreads, TFA were largely substituted by polyunsaturated fatty acids—primarily LI. Soft margarines seemed to have a higher "cardiac risk profile" in 1995 than in 1992, because of a higher content of saturated fatty acids, primarily LA and MY. Soft margarines contained insignificant amounts of *trans*-18:1 in both 1992 and 1995.

A gradual decrease in TFA in margarines has been observed since the mid-1960s in many Western countries, mainly because of improved processing conditions (28). A decline also can be demonstrated for Danish margarines. A total of 30 different Danish margarines, which did not cover the entire retail market, was analyzed in 1985 (29). Margarines with <20% LA, 20–40% LA, and >40% LA had *trans*-18:1 contents of 7.1, 9.8, and 4.1%, respectively. Our

study showed that *trans*-18:1 had decreased to considerably lower levels in 1992, and that a further decrease had occurred in 1995. Compared to the *trans*-18:1 content in 1985, average decreases of 40–90% were seen in margarines analyzed 10 yr later.

A recent study of French tub margarines (semisoft and soft margarines) also demonstrated a decline in *trans*-18:1 content, from a mean of 13% of total fatty acids in 1991 to 3.8% in 1995 (30). In that study, four of the 12 margarines examined were "*trans*-free" (<2% TFA), whereas four years earlier none were "*trans*-free." Incidentally, the French study found extremely high *trans* contents (more than 50% of total fatty acids) in two of the three examined shortenings, much higher than the contents seen in Danish manufactured shortenings.

Shortenings used in the baking industry have a higher *trans* content than comparable (solid) margarines. It is claimed by the margarine manufacturers that the present *trans* content is necessary in shortenings to give the product "unique" physical properties, such as satisfactory mouthfeel and distinctive texture which, to a large extent, characterizes the final food product (e.g., snacks, cakes, biscuits, confectionery). The truth of this claim is, however, scientifically unproven.

Danish shortenings and hard margarines—as is the case for margarines and shortenings produced in the United Kingdom and in other Nordic countries—often contain significant amounts of partially hydrogenated marine oils. Marine oils do not seem to be present at all in margarines with medium and high levels of LI.

The hydrogenation of both vegetable oils and marine oils results in a number of isomers, of which *trans*-monoenoic acids dominate (28). So far, most studies on the effects of TFA have been conducted with partially hydrogenated vegetable oils. A recent study has shown that high intake of TFA (about 20 g/day) from partially hydrogenated marine oils increases total- and LDL-cholesterol and decreases HDL-cholesterol compared to the intake of the same amount of TFA from partially hydrogenated soybean oil (3).

This study showed a sizable reduction from 1992 to 1995 in the use of marine oils in solid margarines, reflected in the reduced content of LC. Shortenings contained a similar amount of LC, about 7% of total fatty acids. A substantial amount of LC was present in the form of *trans*-monoenoic acids (*trans*-20:1, *trans*-22:1). The mean content of *trans*-monoenoic acids from LC constituted almost half of total TFA in shortenings and margarines. Thus, because long-chain *trans*-monoenoic acids are suspected to be atherogenic, there is good reason to urge the margarine industry to continue lowering the content of partially hydrogenated marine oils in shortenings and margarines.

Based on Danish sales figures for margarines and shortenings in 1995 (from the Association of Danish Margarine Manufacturers, which include factories supplying close to 100% of the Danish market), total sale for the Danish market corresponds to a daily per capita supply of 30 g, of which 10 g is made up from shortenings, and 14, 4, and 2 g from hard, semisoft, and soft margarines, respectively. It can be calculated that the average per capita supply of *trans*-18:1 from margarines and shortenings is about 1.5 g daily for every Dane. On the assumption that the sale of margarines was similar in 1992 and 1995, the supply of *trans*-18:1 from margarines has decreased by 0.7 g/day. This decrease has occurred at the same time as saturated fatty acids decreased by 0.7 g/day. This decrease of 1.4 g/day has been replaced by 1.0 g/day of *cis* monounsaturated fatty acids and 0.4 g/day of polyunsaturated fatty acids.

However, it must be remembered that compared to a national average saturated fat intake of 40 g/day (31), the intake of *trans*-18:1 from hydrogenated vegetable oils is indeed very low. Although *trans* content of margarines and shortenings should be minimized, dairy products, margarine, and meats are still the major contributors of saturated fatty acids to the diet and should continue to be the focus of dietary change. Compliance with this recommendation will also appreciably lower dietary TFA.

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Polyunsaturated Fatty Acids Exert Antiarrhythmic Actions as Free Acids Rather Than in Phospholipids

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ABSTRACT: Previous studies have shown that exogenous free n-3 polyunsaturated fatty acids (PUFA) can prevent tachyarrhythmias caused by specific agents in isolated cardiac myocytes. However, the question as to whether incorporation of the n-3 PUFA into membrane phospholipids has the same immediate protective effects remained to be answered. To answer this question, we increased the content of n-3 PUFA in the phospholipids of cultured neonatal rat myocytes by growing them 2–3 d in a culture to which eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) in 15 μM concentration was added. Analysis of the fatty acid composition of membrane phospholipids revealed a significantly higher level of EPA and DHA (from 0.2 to 7.6% and from 1.2 to 6.5%) in cells supplemented with EPA or DHA, respectively. The responses of the myocytes grown in normal media or in media enriched with the PUFA to arrhythmogenic agents were examined after free fatty acids were removed from the medium and the cells. The arrhythmogenic agents used were the β-adrenergic agonist isoproterenol or an elevated extracellular concentration of calcium. The results showed that there was no significant difference in the induction of tachyarrhythmias by isoproterenol or by elevated $[Ca^{2+}]_o$ in cells grown in media enriched with PUFA, as compared with cells grown in normal media in the absence of the free PUFA. Under the conditions of this study, only the unesterified PUFA were able to protect the cardiomyocytes against induced arrhythmias. There was no antiarrhythmic effect due to an increased fraction of EPA or DHA in membrane phospholipids.

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Sudden cardiac death constitutes a major portion (50–60%) of mortality from acute myocardial infarctions, some 250,000 deaths annually in the United States alone (1). Malignant arrhythmias such as ventricular tachycardia and ventricular fibrillation are thought to be main factors causing sudden cardiac death. There are several studies which indicate a role for long-chain n-3 fatty acids in the prevention of fatal ventricular arrhythmias. In experiments with rats and marmosets, McLennan *et al.* (2–4) showed that feeding tuna fish oil, in contrast to saturated animal fat or olive oil, prevented the animals from fatal rhythm disturbances following an experimental myocardial infarction. Similar feeding studies on rats by Hock *et al.* (5) confirmed these results. Billman *et al.* (6) reported that an intravenous infusion of an emulsion comprised largely of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) as their free fatty acids can prevent ischemia-induced ventricular fibrillation in conscious exercising dogs. The animals were prepared by a surgically induced myocardial infarction and placement of a hydraulic cuff around the left circumflex artery, which enabeled subsequent induction of ischemia during exercise. Our earlier studies have shown that free long-chain polyunsaturated fatty acids (PUFA) at 2–15 μM can prevent the arrhythmias induced by ouabain (0.1 mM), high extracellular calcium (7 mM), lysophosphatidylcholine (2–10 μM), acylcarnitine (2-10 μM), or the β-adrenergic agonist isoproterenol (ISO) (2-10 μM) in isolated neonatal rat cardiac myocytes (7–11). In humans a randomized clinical trial has reported a significant reduction in sudden cardiac death in patients whose diets were supplemented with long-chain n-3 fatty acids (12). Another human study showed a reduction in mortality from coronary heart disease without a reduction in heart attacks in patients eating fish, which also suggests a reduction in sudden death (13).

Our previous studies indicate that it is the unesterified free fatty acid which prevents the arrhythmias (7–11). However, it is still unclear—especially with respect to the dietary studies—whether n-3 PUFA in membrane phospholipids can also exert a protective effect. So far, most studies analyzing changes in cell behavior due to effects mediated by the fatty acids focus on the fatty acid composition of the phospholipids in cell membranes. This suggests that some workers feel that it is the resulting enrichment of membrane phospholipid content with the fatty acids that accounts for the changes in cellular functions. In this context, it seemed important to assess whether the antiarrhythmic effect of n-3 PUFA, like EPA and DHA, is based only on effects of their free fatty acid form or if the effect is dependent on incorporation of these fatty acids

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Abbreviations: BSA, bovine serum albumin; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; ISO, isoproterenol; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography.

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into membrane phospholipids. Because dietary feeding studies cannot discern between effects of increased concentrations of PUFA in membrane phospholipids or in the free nonesterified fatty acids, since both increase simultaneously, this study uses isolated multicellular cardiac myocyte cultures to examine the effects of EPA or other n-3 PUFA as a constituent of membrane phospholipids on the inducibility of arrhythmias by isoproterenol (ISO) or high extracellular calcium concentrations. We show that there is no prompt protective effect due to an enrichment of membrane phospholipids with n-3 fatty acids, and that the protective effect of these n-3 PUFA against arrhythmias is only a function of the free, unesterified fatty acid.

METHODS

Cell culture. Cardiac myocytes were isolated from one-day-old rats using the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corp., Freehold, NJ). This system is based on that described by Toraason *et al.* (14), in which minced tissue is incubated overnight with trypsin in the cold. The isolated cells were placed on 15-mm glass coverslips in petri dishes. Cells were cultured at 37°C in air with 5% CO_2 added and 98% relative humidity in a tissue culture incubator (Model 3123; Forma Scientific, Marietta, OH); the medium contained 5% fetal calf serum and 10% horse serum. The culture medium was changed every other day. After 48 h in culture, the myocytes had attached to the coverslips and formed multicellular colonies of regularly and spontaneously beating cells. Cells were used for experiments after 3–5 days of culture.

Fatty acid composition analysis. The fatty acid composition of cellular phospholipids was analyzed as described previously (15). Lipid was extracted with chloroform/methanol (2:1, vol/vol). Samples were sealed under nitrogen and stored at –20°C until thin-layer chromatography (TLC) was performed. For TLC, the samples were dried under nitrogen and reconstituted in 80–100 µL chloroform. TLC plates were activated at 90–100°C for 1 h, and chromatography was run using petroleum ether/ethyl ether/acetic acid (80:20:1, by vol) as the solvent. Bands were made visible with 0.01% 8-anilino-1-naphthalenesulfonic acid, and gel scrapings of the phospholipid fraction were collected for methylation. To obtain fatty acid methyl esters, 1.5 mL borontrifluoride in methanol (14%) and 2 mL hexane were added to the gel scrapings and then heated for one hour at 100°C. After this procedure 1 mL of water was added and mixed vigorously. Tubes were allowed to stand for at least 30 min before removing the hexane phase. Samples were then dried under nitrogen and reconstituted in 100 µL isooctane of which 2 µL were analyzed by gas–liquid chromatography. An Omegawax™ column (30 m; Supelco, Bellefonte, PA) was used in a Hewlett-Packard 5890A gas chromatograph (Hewlett-Packard, Avondale, PA). Carrier gas was hydrogen (2.39 mL/min), injected with a split ratio of 1:31. The initial temperature was 165°C for 5 min; then temperature was increased to 195°C at 2.5°C/min and from there to 220°C at 5°C/min. Temperature was held for 10.5 min and then decreased to 165°C at 27.5°C/min. Peaks were identified by comparison with fatty acid standards (Nu-Chek-Prep, Elysian, MN), and area percentage for all resolved peaks was analyzed using a Perkin-Elmer M1 integrator (Perkin-Elmer, Norwood, CT).

Measurement of cell contraction. Changes in amplitude of contraction and beating rate of cultured cardiomyocytes were determined using a phase contrast microscope and video-monitor edge-detector (Crescent Electronics, Salt Lake City, UT) as described previously (7). A glass coverslip with attached cultured myocytes was brought to a chamber which was continuously superfused during contractility measurements with a HEPES buffered-saline solution (in mM: 140 NaCl, 5 KCl, 1.0 $MgCl_2$, 1.2 $CaCl_2$, 1.0 Na_2HPO_4, 5.0 HEPES, 10 glucose; pH was adjusted to 7.4 with NaOH). In the experiments using high Ca^{2+} concentrations in the perfusion fluid, Na_2HPO_4 was omitted from the buffer to prevent precipitation of calcium salts. The chamber was placed on the stage of a Zeiss Axiovert 10 inverted microscope (Zeiss, Oberkochen, Germany) and was surrounded by a temperature-controlled lucite box heated to 37°C. A four-way manifold controlled the inlet to the perfusion system delivered by two syringe pumps (Harvard Apparatus Inc., South Natick, MA), so that the coverslip could be sequentially superfused with different solutions. The flow rate was 20 mL/h, and the cells were magnified with a 32x objective. Plastic beads added to the cell cultures on day two were used as markers for myocyte motion. The image was monitored with a CCD videocamera (Javelin Electronics, Los Angeles, CA) and displayed on a monitor. A video motion detector system recorded motion along a selected raster line and transferred these data to a Grass 79D strip chart recorder (Grass Instrument Co., Quincy, MA). After 3–5-min equilibration period while perfusing with HEPES buffer, different solutions could be added while observing the contraction behavior of the cells. Changes in amplitude and rate of cell contractions were used to assess the effects of different perfusates on cell contractions.

Statistical analyses. The results of the arrhythmia-induction experiments were analyzed using X^2-test with two degrees of freedom, using 0.05 as level of significance. No corrections were used. The data from the fatty acid analyses are shown as means ± SE, rounded off to the nearest significant figure.

Materials. Neonatal Cardiomyocyte Isolation System Kit was purchased from Worthington Biochemical Corp. ISO was obtained from Elkins-Sinn, Inc. (Cherry Hill, NJ). Fatty acids were obtained from Sigma Chemical Co. (St. Louis, MO). Fatty acids were dissolved weekly in ethanol at the concentration of 10^{-2} M and stored under a nitrogen atmosphere at –20°C. The final concentration of ethanol was negligible and had no effect on myocyte contraction. TLC plates were purchased from Fisher Chemicals (Pittsburgh, PA), as were the standard solvents used in the analysis of fatty acids.

RESULTS

Our goal was to test if there is any change in response of cardiac myocytes to arrhythmogenic agents due to a change in cellular phospholipid content of n-3 PUFA. Therefore, we increased the membrane phospholipid composition of the myocytes with n-3 PUFA. The enrichment of cells with n-3 PUFA was obtained by growing myocytes for at least 48 h in media containing these fatty acid in a concentration of 15 μM. To confirm that an enrichment of phospholipids with n-3 PUFA was achieved, the fatty acid composition of phospholipids was analyzed, as described. The results are shown in Table 1. The analyses revealed a significant increase in the content of EPA from 0.2 ± 0.2 to 7.6 ± 1.0% and in the elongated EPA derivative docosapentaenoic acid (DPA, 22:5n-3) from 2.5 ± 0.9 to 13.1 ± 1.2% in controls vs. EPA-supplemented cells. Cells cultured with DHA showed an increase of DHA in phospholipid from 1.2 ± 0.4 to 6.5 ± 0.3%.

These cells were then used in contractility studies similar to those previously reported (7–11). ISO, as well as a high extracellular calcium concentration, induce spasmodic tachyarrhythmic events in cultured cardiomyocytes, making these cells a suitable model for studying cardiac arrhythmias. The cells, when grown in nonenriched media, show a slowing of contraction rate in response to perfusion with n-3 PUFA (5–15 μM), normally within 3 to 12 min. Perfusion with these fatty acids is able to stop arrhythmias induced by ISO (Fig. 1A) or by an elevated extracellular calcium concentration (Fig. 2A). We compared the response of those cells with that of cells having a higher phospholipid content of long-chain n-3 PUFA (Fig. 1B). After establishing a baseline of contraction while perfusing with HEPES-buffer (3–5 min), 2 mg/mL delipidated bovine serum albumin (BSA) was added to the superfusate for 3 min. Delipidated BSA binds free fatty acids tightly, which removes the free fatty acids carried over from the culture medium and the myocytes. Thereby, an environment essentially free of nonesterified fatty acid was created for the cells.

The cells were then perfused with ISO (3–10 μM) to induce arrhythmias. In both cell groups, controls and PUFA-enriched, we found the induction of arrhythmias by ISO. Of 51 nonenriched cells tested, 26 (51%) developed arrhythmias while perfused with ISO, in comparison to 47 of 107 (44%) EPA-enriched cells and 6 of 13 (46%) DHA-enriched cells. Chi-square tests applied to these data show that there were no significant differences between the two cell groups in terms of induction of arrhythmia by ISO ($X^2 = 0.69$, $P > 0.1$).

In experiments performed with high extracellular Ca^{2+} concentration (7 mM) instead of ISO as arrhythmogenic agent, we induced arrhythmia in 16 of 20 (80%) nonenriched cells tested as compared to 10 of 14 (71%) EPA-enriched cells and 4 out of 6 (67%) cells enriched with DHA ($X^2 = 0.58$, $P > 0.1$). These results indicate that there was no protective effect due to the increased n-3 fatty acids in phospholipid. In all cases tested, perfusion with free EPA or DHA (15 μM) terminated the arrhythmias induced by ISO, both in unenriched controls ($n = 8$) as well as in enriched cells ($n = 23$). When 7 mM extracellular Ca^{2+} was used as arrhythmogenic agent, there was a reversal of arrhythmias by perfusion of EPA or DHA in 10 controls ($n = 12$) as well as in 9 enriched cells ($n = 10$). These data further indicate that the n-3 PUFA in phospholipids had no antiarrhythmic effect in this study. Representative examples for the induction of arrhythmias by ISO and their termination by EPA-perfusion are given for both cell groups in Figure 1. Corresponding data are shown for Ca^{2+}-induced arrhythmias in Figure 2.

These findings show that despite a large enrichment of the n-3 PUFA content in membrane phospholipids of the myocytes, no antiarrhythmic protection was afforded in the absence of free n-3 PUFA. Only free n-3 PUFA were protective against arrhythmias in this study; the same n-3 fatty acids when only present in the membrane phospholipids were not antiarrhythmic.

TABLE 1
Comparison of Phospholipid Fatty Acid Composition in Control Cells and Cells After Growth in Media Containing EPA or DHA (15 μM)[a]

	Phospholipid fatty acid composition (wt%)		
Fatty acid	Control ($n = 5$)	EPA-enriched ($n = 10$)	DHA-enriched ($n = 3$)
16:0	8.8 ± 1.5	7.8 ± 1.4	11.6 ± 0.8
18:0	19.7 ± 2.7	16.2 ± 2.4	15.0 ± 0.9
18:1n-9	9.6 ± 1.2	8.4 ± 0.6	7.8 ± 0.5
18:2n-6	9.9 ± 1.4	9.0 ± 0.9	8.1 ± 0.5
20:3n-6	0.3 ± 0.2	0.4 ± 0.2	1.0 ± 0.0
20:4n-6	13.9 ± 2.3	13.5 ± 1.2	7.7 ± 0.5[b]
20:4n-3	3.7 ± 1.0	2.6 ± 0.4	0.4 ± 0.1
20:5n-3	0.2 ± 0.2	7.6 ± 1.0[b]	1.5 ± 0.1
22:0	0.4 ± 0.4	0.3 ± 0.1	0.5 ± 0.0
22:4n-6	2.1 ± 0.7	1.1 ± 0.4	0.7 ± 0.2
22:5n-3	2.5 ± 0.9	13.1 ± 0.2[b]	1.3 ± 0.1
22:6n-3	1.2 ± 0.4	0.5 ± 0.2	6.5 ± 0.3[b]

[a]Data were obtained by gas chromatography. Values are means ± SE: [b]$P < 0.01$. The minor fatty acids are not included, so the total percentages do not add up to 100; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

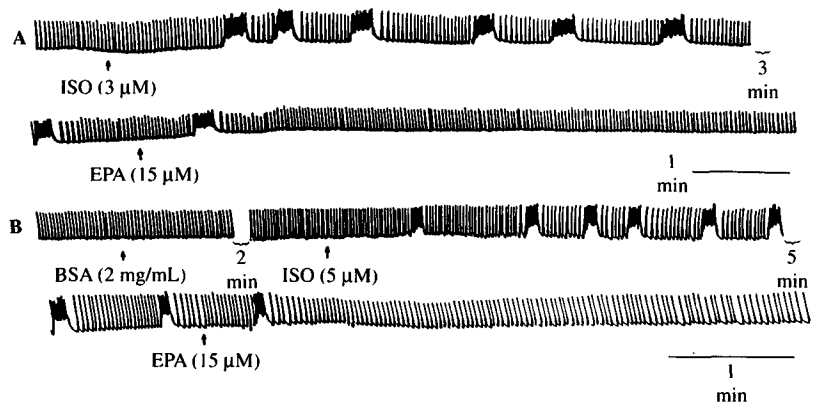


FIG. 1. A. Response of control cells to isoproterenol (ISO). Perfusion with ISO-induced arrhythmias, which could be terminated by subsequent addition of eicosapentaenoic acid (EPA). B. Response of cells enriched with EPA to ISO. Cells were first perfused with buffer, then with bovine serum albumin (BSA) to remove free fatty acids. Perfusion with ISO induced arrhythmia. This arrhythmia could be terminated by additionally perfusing with 15 μM EPA.

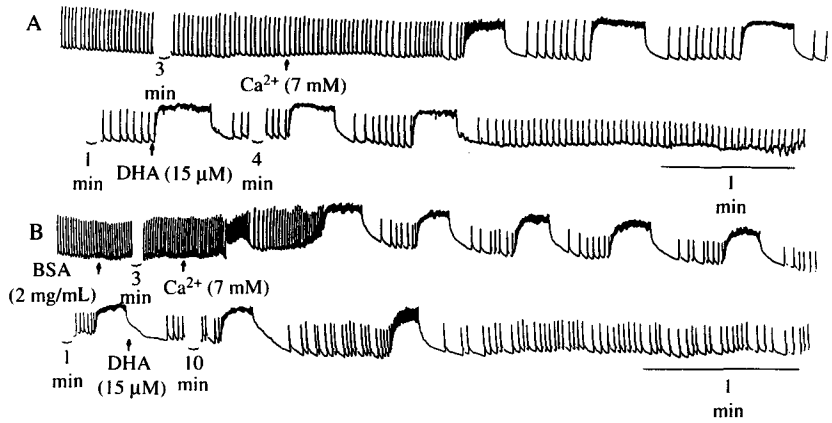


FIG. 2. A. Response of control cell to stimulation with 7 mM extracellular Ca^{2+}. Induced arrhythmias were terminated by subsequent addition of docosahexaenoic acid (DHA) (15 μM). B. Cells cultured in 15 μM DHA showed induction of arrhythmia by 7 mM extracellular Ca^{2+}. Arrhythmia was reversible with perfusion with DHA (15 μM). See Figure 1 for other abbreviation.

DISCUSSION

Many studies examining the effects of fatty acids on myocardial cell function have focused on changes in the fatty acid content of the phospholipids. It seems to be widely assumed that changes in cell functions associated with an increase or reduction of the intake of specific fatty acids result from enrichment or depletion of these fatty acids in cellular phospholipids. This assumption has been implicit in the interpretation of dietary studies that found a correlation between the antiarrhythmic action of n-3 PUFA and their enrichment in membrane phospholipids of cardiac myocytes (2–4). However, these studies do not permit discrimination between a direct effect of the free n-3 PUFA or a direct effect when incorporated in membrane phospholipids, since in animal feeding experiments the free fatty acid levels and the PUFA incorporation in membrane phospholipids both increase simultaneously. The goal of this study was to test the effects of those alternatives separately.

The cell system used consists of an isolated, cultured, spontaneously beating neonatal rat ventricular myocyte preparation with cell-cell communications. These cells respond to ISO stimulation or to stimulation with high extracellular calcium concentrations with development of tachyarrhythmias and chaotic fibrillatory beating activity, thereby showing an arrhythmic profile resembling that recorded from the isolated or *in vivo* heart (16–18). EPA or DHA (5–15 μM) is able to prevent or terminate these arrhythmias when added

to the perfusate (10). Both n-3 and n- 6 PUFA as their free acids are able to exert antiarrhythmic action, in contrast to saturated and monounsaturated fatty acids which have no antiarrhythmic effects (7,10,11) and do not block sodium channels (19) or bind to the channel protein (20) as do the free PUFA. However, the n-6 PUFA arachidonic acid showed induction of arrhythmias in one-third of the experiments (7). This is due to arrhythmogenic cyclooxgenase metabolites derived from arachidonic acid, not to the arachidonic acid *per se*. By using only n-3 PUFA in these experiments, we exclude this possibility, as these PUFA have not been found to be arrhythmogenic in our previous experiments (7,10,11).

An increase in content of n-3 PUFA in phospholipids was established by growing neonatal cardiomyocytes in medium containing EPA or DHA, and these cells were used to test if there is a protective effect arising from n-3 PUFA in membrane phospholipids. By culturing myocytes with or without EPA or DHA added to the medium and then superfusing them with delipidated BSA to remove free EPA or DHA from the medium and the myocytes (7,10), we could test the effect of just the EPA and DHA incorporated into cellular phospholipids in contrast to experiments using the intact animal. Despite the higher content of EPA + DPA (3 vs. 20% in control vs. enriched cells) or of DHA (1 vs. 6.5%) in the phospholipid fraction, there was no protective effect from this enrichment of phospholipids with n-3 PUFA. Furthermore, arrhythmias induced in these cells could be terminated in the same way as observed for cardiomyocytes grown in the absence of the PUFA when the myocytes were subsequently perfused with free EPA (10–15 μM). In this study we saw only a rate of arrhythmia-induction by ISO of 51%, as compared to 100% in a previous study (10), though we have been unable to discern the explanation for this difference.

The observation that it is only free PUFA which has an antiarrhythmic effect is supported by other findings. Studies with Billman showed that intravenous administration of fatty acid emulsions exerted a rapid protective effect against malignant ventricular arrhythmias in dogs without detectable incorporation of the infused n-3 PUFA into plasma phospholipids (Billman, G.E., Kang, J.X., and Leaf, A., unpublished data). Our previous experiments have shown that perfusion of spontaneously beating cardiac myocytes with free n-3 PUFA can terminate arrhythmias in a few minutes, while ethyl esters or triglycerides of these fatty acids fail to do so (6,7). Additionally it was shown that the electrical excitability of the cardiomyocytes can be suppressed by exposing them to free n-3 PUFA for just 2–5 min (19), and in whole-cell voltage clamp studies blocking effects on the sodium current are seen within seconds after the PUFA are applied directly onto the myocytes (21). It has also been shown that PUFA bind specifically to the sodium channel protein (20). In these studies, no comparable effects of the monounsaturated oleic acid or of saturated fatty acids has been noted. Charnock *et al.* (22) have determined the fatty acid composition in normoxic rat hearts. They found that elevating the dietary intake of n-3 fatty acids increased the content of nonesterified fatty acids toward a greater amount of n-3 PUFA in the myocardium. However, because the n-3 PUFA increased in both the nonesterified, free fatty acid fraction and in the phospholipid fraction of the myocardium, they were unable to distinguish in which fraction the n-3 PUFA were exerting their antiarrhythmic action.

When the myocytes are enriched in their phospholipid content of n-3 PUFA, conditions that will activate cellular phospholipases may liberate the PUFA as free fatty acids. The PUFA esterified in phospholipids could thus contribute indirectly and secondarily to an antiarrhythmic effect. This study does not test this possibility. This study does, however, indicate that it is only the free PUFA which exerts a protective effect against arrhythmias. The alternative that the effect of PUFA against arrhythmias is a direct effect of their incorporation into phospholipids can be excluded. It is only the free PUFA partitioning nonconvalently among the membrane lipids of cardiac myocytes which exert an antiarrhythmic effect.

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Enhancement by Ganglioside GT1b of Annexin I Phosphorylation in Bovine Mammary Gland in the Presence of Phosphatidylserine and Ca^{2+}

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ABSTRACT: Ganglioside GT1b and, to a lesser extent, GD3, enhanced phosphorylation of a 36 kDa protein (the substrate of protein kinase C) in the particulate fraction from bovine mammary gland. Sialic acids, asialogangliosides, and GM3 were without effect, and GD1a conversely inhibited phosphorylation of the 36 kDa protein. The enhanced phosphorylation by GT1b required the simultaneous presence of phosphatidylserine (PS) and Ca^{2+}. The 36 kDa protein reacted with anti-annexin I in Western blot analysis. Addition of purified annexin I to the reaction mixture containing the particulate fraction increased the extent of phosphorylated 36 kDa protein, and the phosphorylation was further enhanced by GT1b. The enhanced phosphorylation of annexin I by GT1b was also dependent on PS and Ca^{2+}. When annexin I was phosphorylated by purified protein kinase C, GT1b inhibited the annexin I phosphorylation. Addition of epidermal growth factor or insulin to the particulate fraction had little effect on the enhancement. These results suggest that an enzyme or enzymes other than protein kinase C, epidermal growth factor receptor kinase, or insulin receptor kinase is responsible for the GT1b- and GD3-enhanced phosphorylation of annexin I in the presence of PS and Ca^{2+}.
Lipids 31, 983–987 (1996).

Annexins belong to a family characterized by the ability to bind with membrane phospholipids in a Ca^{2+}-dependent manner, and are distributed in a wide variety of cells and tissues (1,2). Annexin I, best characterized in the family, is suggested to be implicated in the regulation of various biological processes, including exocytosis (2–4), eicosanoid production (5), and synthesis and secretion of prolactin (6). In the mammary gland, this protein is reported to be related to the mammary cell differentiation (7). Annexin I is the substrate for protein kinase C (PKC; 8), and phosphorylation by PKC suppresses the ability of this protein to aggregate phospholipid vesicles (3,4).

Gangliosides, localized mainly in the outer surface of the plasma membrane, modulate the activities of various protein kinases, including PKC. Gangliosides inhibit PKC activity when histone is used as the substrate, and the order of inhibitory capacity is dependent on the number of sialic acid residues (9). By comparison, in the case of endogenous protein phosphorylation, gangliosides act as inhibitors or stimulators for individual substrates. In bovine mammary gland, phosphorylation of most endogenous substrates for PKC is inhibited by the hydrophilic gangliosides such as GT1b, whereas some substrate phosphorylation is resistant to GT1b inhibition (10). In particular, phosphorylation of a 36 kDa protein present in the particulate fraction is enhanced by GT1b. In the present study, we show that phosphorylation of the 36 kDa protein, which was identified as annexin I, is enhanced by GT1b in the presence of phosphatidylserine (PS) and Ca^{2+}.

MATERIALS AND METHODS

Materials. Gangliosides and related compounds were purchased from Sigma Chemical Co. (St. Louis, MO). Monoclonal antibodies to bovine lung annexins I, II, IV, and VI were from Affiniti Research Products (Nottingham, United Kingdom). Recombinant human epidermal growth factor (EGF) was bought from Wako Pure Chemicals (Osaka, Japan). [γ-^{32}P]ATP was obtained from DuPont-New England Nuclear (Wilmington, DE). Cow mammary glands in the lactating state were collected from a local slaughterhouse.

Phosphorylation of total particulate fraction. The total particulate fraction from bovine mammary gland was prepared, and phosphorylation of endogenous proteins was carried out as described (11). Briefly, the standard assay mixture (0.2 mL) contained 25 mM *tris*(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.5), 10 mM $MgCl_2$, 0.25 mM EGTA, 10 mM 2-mercaptoethanol, 50 μM [γ-^{32}P]ATP, and 20 μL of the particulate fraction (containing 40–50 μg of protein), in the absence or presence of 1-oleoyl-2-acetyl-*sn*-glycerol (OAG; 1 μg, 12.5 μM), PS (5 μg, 32 μM), and Ca^{2+} (1 μM). Gangliosides were sonicated in 20 mM Tris-HCl (pH 7.5), then incubated in the mixture. The reaction was initiated by the addition of ATP and continued for 5 min at 30°C. Phos-

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Abbreviations: EGF, epidermal growth factor; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PDGF, platelet-derived growth factor; PKC, protein kinase C; PS, phosphatidylserine; Tris, *tris*(hydroxymethyl)-aminomethane.

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phorylated proteins were separated by SDS–polyacrylamide gel electrophoresis, using 12 or 7.5% gels, then visualized by autoradiography.

Purification of annexin I. Annexin I from bovine mammary gland was purified essentially according to the method of Wang and Creutz (3). Briefly, the minced tissue (200 g) was homogenized using a Polytron for 1 min with 2 vol of buffer A (20 mM Tris-HCl, pH 7.5, 150 mM KCl, 0.12 TIU/mL aprotinin, 1 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1 mM benzamidine, and 4 mM iodoacetic acid), and centrifuged at 2,900 × *g* for 15 min. To the supernatant, $CaCl_2$ was added to a final concentration of 2 mM. The pellet obtained by centrifugation at 105,000 × *g* for 30 min was redissolved in buffer A (from which iodoacetic acid was deleted) containing 10 mM EGTA. Annexin I was further purified by sequential chromatographies with DEAE-Sephacel, hydroxylapatite, and Mono S. Annexin I thus obtained was homogeneous as revealed by SDS–polyacrylamide gel electrophoresis, but a faint additional band (33–34 kDa) was occasionally detected by Western blot analysis and autoradiography. In the blotting, both annexin I (36 kDa) and the 33–34 kDa protein reacted with anti-annexin I, but not with anti-annexins II, IV, and VI, thereby suggesting that the band was a breakdown product of annexin I.

Other methods. PKC was purified from the mammary gland by ammonium sulfate fractionation and DEAE-cellulose, Sephacryl S-200, and protamine-Sepharose 4B chromatographies, and phosphorylation of annexin I by PKC was done as described previously (11). Phosphorylation of the particulate proteins with annexin I added in the presence of EGF or insulin was performed as reported (12,13). All phosphorylation experiments were repeated using 2–5 mammary samples from different cows. Phosphoprotein patterns obtained using different samples were essentially similar, but individual variations of values obtained by densiometer scanning of autoradiograms were too large to analyze statistically. Autoradiograms were scanned using Bio-Profil (Vilber Lourmat, Marnelavalleé, France). Duplicate or triplicate scans were done for each lane, variations between scans being less than 10%. Protein was determined by the method of Bradford (14).

RESULTS

The total particulate fraction from bovine mammary gland contained several PKC substrates phosphorylated in the presence of the PKC cofactors OAG, PS, and Ca^{2+} (Fig. 1). These included 94 kDa, 91 kDa, 56 kDa, 42 kDa, and 36 kDa proteins. A 30 kDa protein, whose phosphorylation was independent of the cofactors, was casein. Although phosphorylation of these particulate substrates was relatively resistant to ganglioside inhibition, compared with that of cytosolic proteins (10), GT1b inhibited phosphorylation of the 91 kDa protein. Phosphorylation of the 36 kDa protein was inhibited by GD1a, but was enhanced by GD3 and in particular by GT1b. GM3 had little effect on the 36 kDa phosphorylation. *N*-acetylneuraminic acid, *N*-glycolylneuraminic acid, asialoGM1, and asialoGM2 also had little effect up to a 100 μM concentration (figure not shown). Sphingosine, like GD1a, inhibited the 36 kDa phosphorylation (11). Other than the 36 kDa protein, the 56 kDa protein phosphorylation was enhanced by GD3. However, phosphorylation of the 56 kDa and 42 kDa proteins was sometimes detected independent of the cofactors as shown in Figure 2.

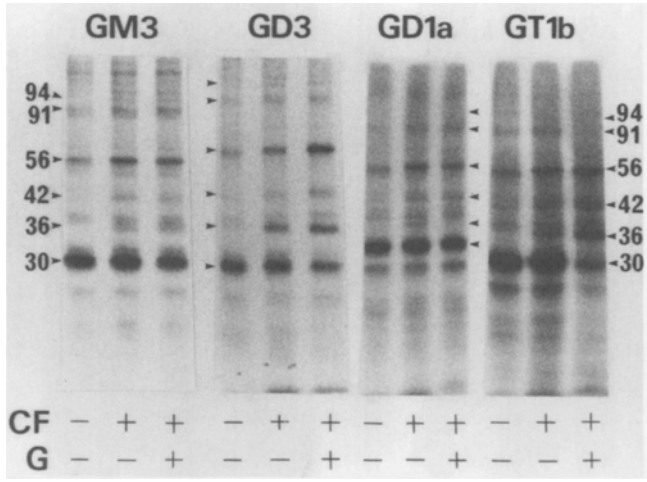


FIG. 1. Effects of gangliosides GM3, GD3, GD1a, and GT1b on phosphorylation of endogenous proteins from the total particulate fraction of bovine mammary gland. CF, protein kinase C cofactors (5 μg/mL 1-oleoyl-2-acetyl-*sn*-glycerol, 25 μg/mL phosphatidylserine, and 1 μM Ca^{2+}); G, ganglioside (100 μM).

The enhanced phosphorylation by GT1b of the 36 kDa protein was dependent on the ganglioside concentrations (Fig. 2, lanes 1–5). The ED_{50} value was estimated to be approximately 10 μM GT1b. With 50 μM GT1b, 2.1- to 5.6-fold increases of the 36 kDa protein phosphorylation were observed by densitometer tracing (the results of five independent experiments). The enhanced effect of GD3 was less potent than that of GT1b (with 50 μM GD3, the increases were 1.3- to 1.8-fold).

Because of its molecular weight, its distribution in the particulate fraction and phosphorylation by PKC, the 36 kDa protein was assumed to be annexin I. Western blot analysis using anti-bovine annexin I revealed that the antibody reacted with the 36 kDa protein (Fig. 3). Antibodies to annexins II, IV, and VI did not react with the protein. Purified annexin I was included in the reaction mixture together with the particulate fraction. The phosphorylation of annexin I observed was dependent on the PKC cofactors (Fig. 2, lanes 6 and 7), and the phosphorylated annexin I band overlapped that of the 36 kDa protein. This result, taken together with that shown in Figure 3, indicated that the 36 kDa protein was annexin I. GT1b had little effect on the annexin I phosphorylation in the absence of the cofactors (Fig. 2, lane 8), but when present, GT1b further enhanced the phosphorylation (1.4- to 1.6-fold, lanes 7 and 9).GD3 also enhanced annexin I phosphorylation in the presence of the cofactors (figure not shown).

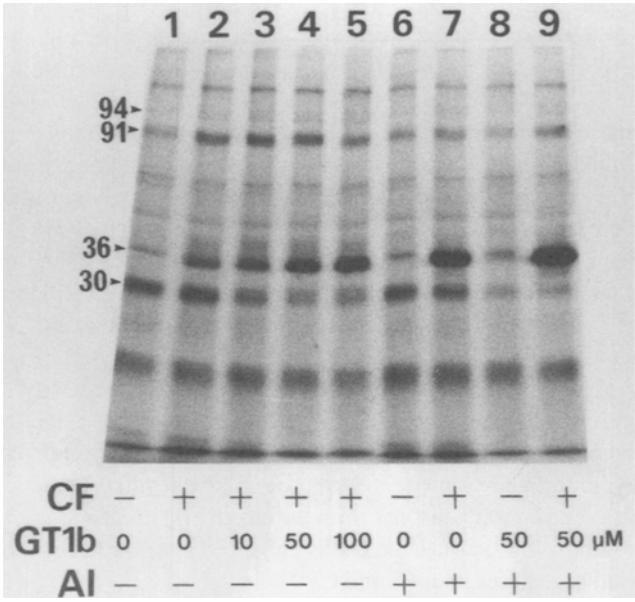


FIG. 2. Effect of GT1b on phosphorylation of the 36 kDa protein and added annexin I. Annexin I (AI, 4.76 μg) was included in the reaction mixture containing 20 μL of the particulate fraction, and phosphorylation was carried out in the absence or presence of the protein kinase C cofactors (CF; 5 μg/mL 1-oleoyl-2-acetyl-*sn*-glycerol, 25 μg/mL phosphatidylserine, and 1 μM Ca^{2+}) and/or various concentrations of GT1b.

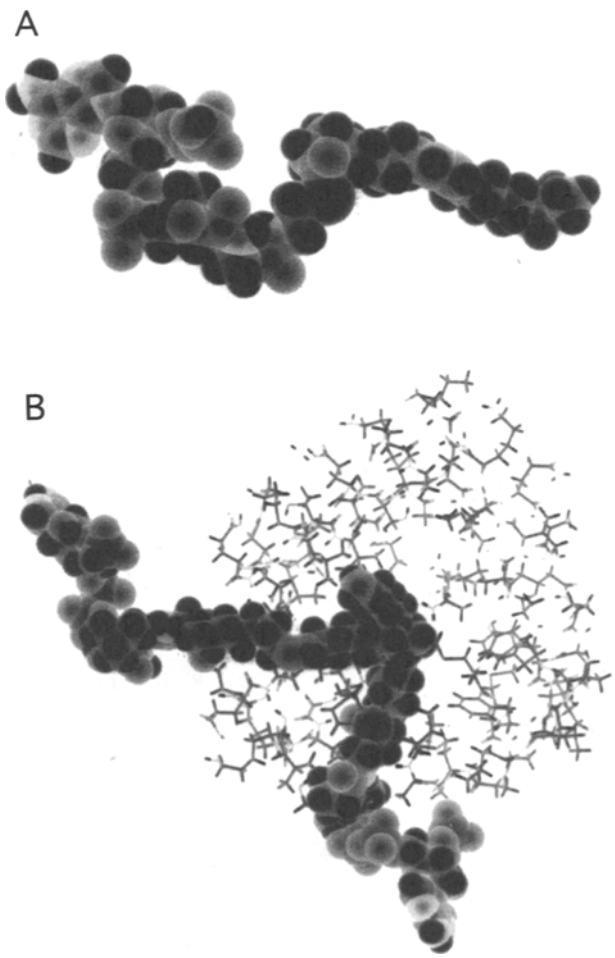


FIG. 3. Western blot analysis of the 36 kDa particulate protein using anti-annexin I. Lanes 1–2, autoradiography; lane 3, the blotting: CF, the protein kinase C cofactors (5 μg/mL 1-oleoyl-2-acetyl-*sn*-glycerol, 25 μg/mL phosphatidylserine, and 1 μM Ca^{2+}).

Of the three PKC cofactors, the simultaneous presence of PS and Ca^{2+} was required for the enhanced phosphorylation by GT1b of annexin I (Table 1). OAG appeared not to be necessary for the GT1b-enhanced phosphorylation. PS and Ca^{2+}, used singly or in combination with OAG, showed little effect on the enhancement by GT1b. Higher concentrations of PS (250 μg/mL) or Ca^{2+} (250 μM), also added separately or in combination with OAG (50 μg/mL), had little effect (data not shown).

To identify the enzyme(s) responsible for the enhanced phosphorylation of annexin I by GT1b, purified annexin I was phosphorylated by PKC in the presence of GT1b (Fig. 4). Phosphorylation of annexin I in the presence of cofactors (lane 2) was inhibited by GT1b in a dose-dependent manner (lanes 3–5). The suppressed phosphorylation by GT1b was overcome by the addition of the higher concentration of PS, but not or only slightly by OAG or Ca^{2+} (lanes 6–8). GM3 and GD3 also inhibited the annexin I phosphorylation, and these inhibitions were similarly reversed by PS (figure not shown). Other than PKC, annexin I is phosphorylated by EGF receptor kinase (12) and insulin receptor kinase (13). The addition of EGF (100 ng/0.1 mL) or insulin (10^{-7} M) did not result in a change of the extent of annexin I phosphorylation in the absence or presence of the PKC cofactors and/or GT1b. Stimulation by these ligands of autophosphorylation of EGF receptor (170 kDa) and insulin receptor (95 kDa) was not clearly observed (figure not shown). GT1b, used singly or in combination with EGF or insulin, also showed little effect on the autophosphorylation.

TABLE 1
Effects of 1-Oleoyl-2-Acetyl-*sn*-Glycerol (OAG), Phosphatidylserine (PS), and Ca^{2+} on Phosphorylation of Annexin I in the Presence of GT1b[a]

OAG (μg/mL)	PS (μg/mL)	Ca^{2+} (μM)	GT1b (μM)	Relative density (%)
0	0	0	0	41
5	25	1	0	100
0	0	0	50	39
5	25	1	50	210
5	0	0	50	32
0	25	0	50	29
0	0	1	50	36
5	25	0	50	46
5	0	1	50	39
0	25	1	50	178

[a]Annexin I (3.62 μg) was included in the reaction mixture (0.2 mL) containing 20 μL of the particulate fraction, and was phosphorylated in the absence or presence of the protein kinase C cofactors and/or GT1b as indicated. After autoradiography, the band corresponding to added annexin I plus the endogenous 36 kDa protein was scanned by a densitometer.

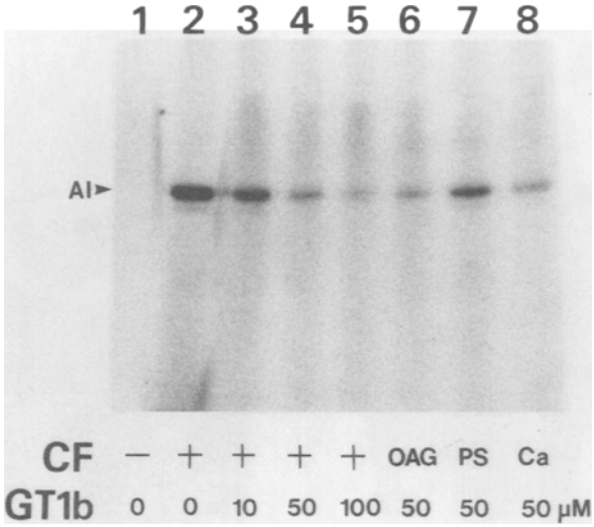


FIG. 4. Inhibition by GT1b of phosphorylation of annexin I by protein kinase C (PKC) and its reversal by the PKC cofactors. Annexin I (3.17 μg) was phosphorylated by PKC [9.7 U (pmol/min)] for 5 min at 30°C. CF, 5 μg/mL 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), 25 μg/mL phosphatidylserine (PS), and 1 μM Ca^{2+}; OAG, 50 μg/mL OAG, 25 μg/mL PS, and 1 μM Ca^{2+}; PS, 250 μg/mL PS, 5 μg/mL OAG, and 1 μM Ca^{2+}; Ca, 250 μM Ca^{2+}, 5 μg/mL OAG and 25 μg/mL PS. Lane 1 contains PKC alone.

DISCUSSION

The present study indicated that phosphorylation of a 36 kDa PKC substrate in the particulate fraction from bovine mammary gland, identified as annexin I, was enhanced by GT1b. Of the gangliosides and the related compounds tested, GT1b and, to a lesser extent, GD3 enhanced annexin I phosphorylation, whereas GD1a and sphingosine inhibited it. Sialic acids, asialogangliosides, and GM3 showed little effect on the enhancement, suggesting that the enhanced phosphorylation was not simply caused by nonspecific binding of sialic acids, but was attributable to the interaction of protein kinase(s) or annexin I with the specific gangliosides, including GT1b and GD3.

The enzyme(s) responsible for the enhanced phosphorylation is unknown at present. Because PS and Ca^{2+} are required, PKC was initially postulated as a candidate. However, purified annexin I phosphorylation by PKC was inhibited by GT1b. Rat mammary gland contains PKC isozymes α, β1, β2, γ, and ζ, in which α is predominant (15). It is unlikely that ζ (atypical PKC) or new PKC are involved in the GT1b-enhanced phosphorylation of annexin I because these isozymes are Ca^{2+}-independent (16). Annexin I may be sequentially phosphorylated, i.e, initially phosphorylated by PKC, after which enhanced phosphorylation by GT1b is catalyzed by an enzyme or enzyme(s) such as Ca^{2+}/phospholipid-dependent protein kinase, which are reported to be distinct from PKC (17).

Annexin I is also the substrate for EGF receptor kinase (12) and insulin receptor kinase (13), and gangliosides modulate these tyrosine kinase activities (18,19). Phosphorylation of annexin I was not induced by EGF or insulin. Because bands corresponding to autophosphorylation of EGF and insulin receptors were not detected, these kinase activities may be defective in this tissue or the assay conditions might be inadequate for the kinases distributed in this tissue. In any case, EGF receptor kinase and insulin receptor kinase are suggested not to be involved in the GT1b-enhanced phosphorylation of annexin I. Tyrosine kinase activities of nerve growth factor receptor (20) and platelet-derived growth factor (PDGF) receptor (21) are also regulated by gangliosides. In the latter case, GT1b is one of the most effective gangliosides in inhibiting autophosphorylation of PDGF receptor (21). GT1b has been also shown to activate several protein kinase activities. Highly sialylated gangliosides, including GT1b, activate protein kinase activity in neuroblastoma cells in a Ca^{2+}-dependent manner (22). GT1b stimulates phosphorylation of several endogenous proteins in myelin by a Ca^{2+}-independent protein kinase (23). These kinases appear to be distinct from the enzyme(s) discussed in the present study, because the GT1b-enhanced annexin I phosphorylation requires both PS and Ca^{2+}.

Annexins I and II are secreted by a variety of cells (24,25). Extracellular annexins I and II are suggested to mediate the anti-inflammatory effect of glucocorticoids (5,25). Cell surface annexin II has been recently found to be a receptor for tanascin-C (26), which is prominently expressed in developing tissues. GT1b may enhance phosphorylation of annexin I on the external cell surface. The presence of extrinsic protein kinases, for example, PKC (27), has been already reported.

GT1b is the minor ganglioside species in bovine mammary gland, while GD3 is abundantly present (28,29). Because phosphorylation of annexin I is involved in membrane fusion processes (3,4), it is presumed in mammary gland that cell surface annexin I participates in the secretion of milk proteins, and that the secretory system may be regulated by phosphorylation by PKC, and further by GT1b- or GD3-modified phosphorylation. The GT1b-enhanced phosphorylation of annexin I is also implicated in the synthesis of milk proteins, because GT1b, GD3, and annexin I, as well as PKC, are found in the nuclear fraction from lactating bovine mammary gland (29–31), and also because rat mammary annexin I is involved in cell differentiation (7). Concentration changes of annexin I and gangliosides localized on cell surface and in nucleus have not yet been evaluated during the lactation cycle. Moreover, effects of steroid hormones and prolactin on their concentrations are not known. Studies along these lines should elucidate functional role(s) of the GT1b-enhanced annexin I phosphorylation in bovine mammary gland.

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Sterol Composition of *Phaeodactylum tricornutum* as Influenced by Growth Temperature and Light Spectral Quality

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ABSTRACT: In a detailed sterol analysis of the marine diatom *Phaeodactylum tricornutum,* free sterols as well as esterified and glycosylated conjugates were found. When the alga was grown under standard conditions (i.e., at 13°C under white light), 64% of total sterols were steryl glycosides. In all sterol classes, except steryl esters, (24*S*)-24-methylcholesta-5,22*E*-dien-3β-ol (epibrassicasterol) was the major (80 to 99%) sterol component. Eight other sterols were identified. Growth under different light spectral quality (red, blue, yellow, and green) at 13 and 23°C was examined. At 23°C, a dramatic decrease in total sterol content was observed, especially under blue light. The distribution of sterols between free and conjugated forms as well as sterol profile inside each class was found to be strongly dependent on the light spectral quality at both temperatures.

Lipids 31, 989–994 (1996).

Cultures of microalgae are extensively used as food for the commercial rearing of marine invertebrates such as oysters. Diatoms, which are abundant components of the phytoplankton, constitute a major nutritional source in aquaculture (1). As dietary polyunsaturated fatty acids and sterols are of great importance for growth and development of marine invertebrates, investigations have focused on these compounds. *Phaeodactylum tricornutum*, a marine pennate diatom (class Diatomophyceae), can satisfy these dietary requirements. Polar lipids of this diatom are characterized by a high content in C_{20}-polyunsaturated fatty acyl chains, mainly 20:5n-3, which is a major compound in stationary phase (2,3). The biosynthetic pathways of these polyunsaturated fatty acids in *P. tricornutum* have been recently investigated (4,5). Sterols are also an important aspect of lipids in marine nutrition. Bivalve mollusks are unable to synthesize *de novo* the sterols they need as membrane constituents and precursors of steroid derivatives (6), but are capable of using sterols from dietary sources such as phytoplankton (7). Few reports have dealt with sterols of *P. tricornutum* (3,8,9). The major sterol, which accounted for 91% of total sterols, was 24-methylcholesta-5,22-dien-3β-ol (3), with an α-oriented (or 24*S*) methyl group at C-24 of the side chain (i.e., epibrassicasterol) (8). The occurrence of 9% Δ7-sterols was reported (3), but has not been confirmed (9). We therefore decided to reinvestigate the sterol composition of *P. tricornutum*, with special emphasis on steryl conjugates. Moreover, since conditions of algal growth can affect its sterol composition (9 and references cited), we examined the influence of light spectral quality and temperature on the sterol profile of *P. tricornutum*. We show that this diatom contains free sterols (FS) as well as steryl esters (SE), steryl glycosides (SG), and acylated steryl glycosides (ASG). These different sterol classes were analyzed for their sterol composition. The nature of sugar(s) and fatty acyl chains of steryl conjugates was not determined. Our results indicate that profound alterations in the total amount as well as in the sterol profile of each sterol class of *P. tricornutum* occur in response to changes in both light spectral quality and culture temperature.

MATERIALS AND METHODS

Algal material and culture conditions. A unialgal culture of the diatom *P. tricornutum* Bohlin was obtained from the Culture Collection of the Laboratoire de Biologie et Biotechnologies Marines (University of Caen Basse-Normandie, France) (10). Under our growth conditions, this strain consists in colonial fusiform cells, with rare single or triradiate cells. A 150 mL-aliquot of stock culture was transferred in 2.5 L of enriched (soil extract and vitamins) sea water medium ES-Tris II (11) supplemented with 0.2 mM sodium metasilicate in 4-L flasks under aseptic conditions. Cultures were incubated at 13 or 23°C (±0.5) with a 12/12 h light/dark cycle provided from cool white, red, blue, yellow, and green 60 W lamps (Osram) at a light intensity of 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ (12). All cultures were bubbled with 0.22 μm-filtered air. Cells were harvested at the beginning of stationary phase by centrifugation and lyophilized.

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Abbreviations: ASG, acylated steryl glycosides; FS, free sterols; GC, gas chromatography; MS, mass spectrometry; SE, steryl esters; SG, steryl glycosides.

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Sterol analysis. Sterols were extracted from dried samples with dichloromethane/methanol (2:1, vol/vol). After solvent evaporation, lipid extracts were applied to silica gel plates (0.25 mm, Durasil-25UV$_{254}$; Macherey-Nagel, Düren, Germany) to separate FS, SE, SG, and ASG (13). Sterols from SE, SG, and ASG were obtained as previously described (12,13). After acetylation, FS and sterols from conjugates were identified by gas chromatography (GC) using a chromatograph equipped with a flame-ionization detector and a glass fused silica capillary column (30 m × 0.32 mm) coated with a 0.25 μm film of CP-Sil5 (Chrompack, Middleburg, The Netherlands). Column was operated at head pressure of 10 psi of helium. The oven temperature was raised from 260 to 300°C at 2°C/min and held at 300°C for 10 min. Injector and detector temperatures were held at 310°C. Sterol identities were confirmed by GC/mass spectrometry (MS) (14,15) and quantified by GC using 5α-cholestane as an internal standard.

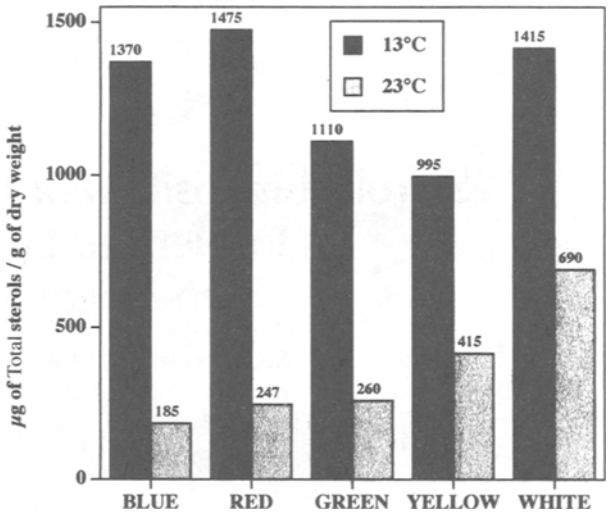


FIG. 1. Total sterol content in *Phaeodactylum tricornutum* grown at 13 and 23°C under different light spectral qualities.

RESULTS

Effects of light spectral quality and growth temperature on total sterol content. In Figure 1 are shown total sterol amounts of *P. tricornutum* grown at 13 and 23°C under different light spectral qualities. When expressed as μg/g dry weight, the total sterol content of cells grown at 13°C appears to be roughly similar (between 1100 and 1500) in all the illumination situations, with slightly lower amounts for cells grown under yellow and green light.

When grown at 23°C instead of 13°C, a dramatic decrease in total sterol amounts of cells was found, especially under blue light, with a 7-fold reduction. Such a temperature shift triggered a net inhibition of sterol biosynthesis, which was observed not to slow down the cell growth (data not shown). A similar but less important (1.4- to 3.7-fold) decrease in the total sterol content in cells of the chrysophyte *Ochromonas danica* was also reported to occur for growth temperature above 23°C (16).

Analysis of the different sterol classes. When grown at 13°C under white light, *P. tricornutum* was found to contain FS, but also SE, SG, and ASG. In contrast to most higher plant cells, FS were not the major compounds. The predominant sterol class was SG with 64% of total sterols (Table 1). FS represented 33% and ASG and SE together, only 3%. Such a repartition of sterols between the different classes was

TABLE 1
Distribution of Free and Conjugated Sterols in *Phaeodactylum tricornutum* Grown at 13 and 23°C Under Different Light Illuminations[a]

		Growth temperature							
		13°C				23°C			
		Sterol composition				Sterol composition			
Light spectral quality		FS	SE	SG	ASG	FS	SE	SG	ASG
Blue	%	20	2	76	2	73	12.5	5.5	9
	μg/g	275	30	1040	25	135	25	10	15
Red	%	60	2	22	16	80.5	14.5	4	1
	μg/g	885	30	325	235	200	35	10	2
Green	%	25	3.5	51	20.5	8.5	36	41	14.5
	μg/g	275	40	565	230	20	95	110	35
Yellow	%	35	7	18	40	34	14	50	2
	μg/g	350	70	180	395	140	60	205	10
White	%	33	0.5	64	2.5	45	2.5	31	21.5
	μg/g	470	5	905	35	310	15	215	150

[a]FS, free sterols; SE, steryl esters; SG, steryl glycosides; ASG, acylated steryl glycosides.

found to be strongly dependent on the type of illumination. Whereas SG remained the main sterol conjugates under green and blue light, a marked increase in FS was observed under red light and in ASG under yellow light. In all situations, SE were minor forms of sterols.

When the culture temperature was raised to 23°C, a completely different distribution of sterols was observed. Under white light as well as under red and blue light, FS were the major compounds. SG were found to represent 50% of total sterols under yellow light and similar amounts of SG and SE (41 and 36%, respectively) were present in cells grown under green light.

Effects of light spectral quality and growth temperature on the sterol composition of each class. Sterol analysis. Nine sterols were identified in *P. tricornutum*. The major compound was by far 24-methylcholesta-5,22-dien-3β-ol (**4**) as already reported (3,9). The configuration at C-24 of this sterol was previously assigned to 24α (or 24*S*) (8); this compound therefore corresponding to epibrassicasterol. The second major sterol was found to be cholest-5-en-3β-ol (cholesterol) (**2**). Sterols with an ethyl group at C-24 such as (24ξ)-24-ethylcholest-5-en-3β-ol (**8**) and (24ξ)-24-ethylcholesta-5,22*E*-dien-3β-ol (**7**) were present in very low amounts, indicating that in *P. tricornutum* the enzyme involved in the second methylation step was not very active. Minor sterols, 5α-cholestan-3β-ol (cholestanol) (**3**), cholesta-5,22*E*-dien-3β-ol (22-dehydrocholesterol) (**1**), (24ξ)-24-methylcholesta-5,24(24^1)-dien-3β-ol (24-methylenecholesterol) (**5**), (24ξ)-24-methylcholest-5-en-3β-ol (**6**), and 24-ethylcholesta-5,24(24^1)*Z*-dien-3β-ol (isofucosterol) (**9**), were also identified in agreement with previous data (9). The C-24 stereochemistry was not determined in this work, but as *P. tricornutum* belongs to the order Pennales and contains both epibrassicasterol and isofucosterol, two sterols with a 24α-oriented alkyl group, all other 24-alkylsterols of this diatom likely have the same configuration (17). Diatoms from the order Pennales would be unique among algae, since most other algae contain sterols with a 24β-configuration (18).

Effects of light spectral quality at 13°C (Tables 2 to 6). Whatever the spectral nature of light illumination, epibrassicasterol (**4**) was the major compound of FS (between 68 and 95% of total FS). Cholesterol (**2**) accounted for only 2 to 5%, except for cells grown under red light (25%). Such cells were also found to contain 7% (24ξ)-24-ethylcholesta-5,22*E*-dien-3β-ol (**7**).

SE were characterized by a lower content in epibrassicasterol (**4**) compared to FS. Cholesterol (**2**) was the major sterol in SE for cells grown under white and blue light and 22-dehydrocholesterol (**1**), the major one under yellow and green light. Significant amounts of 24-ethylsterols were present under all light conditions.

SG and ASG were mainly constituted of epibrassicasterol (**4**), except for ASG from cells grown under red light in which cholesterol (**2**) was predominant. The C-24 alkylated sterols (**6**), (**7**), (**8**), and (**9**) were not detected as glycosylated conjugates, but other compounds with similar relative retention times have been found. They are designated as "unknown" in Tables 2 to 6. According to MS data, these compounds might correspond to hydroxylated derivatives of epibrassicasterol and cholesterol. However, conclusive determination of their structure requires nuclear magnetic resonance analysis.

Whatever the light spectral quality, 24-methylenecholesterol (**5**), a biosynthetic intermediate in the synthesis of epibrassicasterol (8), was never detected as a free compound.

Effects of light spectral quality at 23°C (Tables 2 to 6). In FS, epibrassicasterol (**4**) remained the major sterol, except for cells grown under green light. Cholesterol (**2**) was the second best represented compound.

Cholesterol (**2**) was present as the main sterol in SE. Significant amounts of 24-methylenecholesterol (**5**), were also detected.

The sterol composition of SG was dominated by epibrassicasterol in cells grown under white, red, green, and yellow light, but by cholesterol, under blue light. In ASG, epibrassicasterol (**4**), cholesterol (**2**), and 24-methylenecholesterol (**5**) were the major compounds.

TABLE 2
Relative Sterol Composition of Free and Conjugated Forms of Sterols in *Phaeodactylum tricornutum* Grown at 13 and 23°C Under Blue Light

	Growth temperature							
	13°C				23°C			
Sterol	FS	SE	SG	ASG	FS	SE	SG	ASG
Cholesterol (**2**)	5[a]	63	3.5	13	27.5	86.5	70.5	44
Epibrassicasterol (**4**)	89	9	95	48	66.5	2.5	12	35.5
24-Methylenecholesterol (**5**)	—	8.5	—	5	—	6	6.5	10.5
(24ξ)-24-Methylcholest-5-en-3β-ol (**6**)	—	—	—	—	0.5	—	—	—
(24ξ)-24-Ethylcholesta-5,22*E*-dien-3β-ol (**7**)	—	10	—	—	3.5	—	—	—
(24ξ)-24-Ethylcholest-5-en-3β-ol (**8**)	3.5	4	—	—	2	5	—	—
Isofucosterol (**9**)	2.5	6.5	—	—	—	—	—	—
Unknown	—	—	1.5	34	—	—	11	10

[a]Percentage of total sterols in each sterol class; —, not detected; trace amounts (<0.5%); Retention times of steryl acetates relative to 5α-cholestane/cholesta-5,22*E*-dien-3β-ol (22-dehydrocholesterol) (**1**) = 1.57; cholest-5-en-3β-ol (cholesterol) (**2**) = 1.64; 5α-cholestan-3β-ol (cholestanol) (**3**) = 1.67; (24*S*)-24-methylcholesta-5,22*E*-dien-3β-ol (epibrassicasterol) (**4**) = 1.75; 24-methylcholesta-5,24(24^1)-dien-3β-ol (24-methylenecholesterol) (**5**) = 1.80; (24ξ)-24-methylcholest-5-en-3β-ol (**6**) = 1.86; (24ξ)-24-ethylcholesta-5,22*E*-dien-3β-ol (**7**) = 1.97; (24ξ)-24-ethylcholest-5-en-3β-ol (**8**) = 2.09; [24(24^1)*Z*]-24-ethylcholesta-5,24(24^1)-dien-3β-ol (isofucosterol) (**9**) = 2.15.

TABLE 3
Relative Sterol Composition of Free and Conjugated Forms of Sterols in *Phaeodactylum tricornutum* Grown at 13 and 23°C Under Red Light

	Growth temperature							
	13°C				23°C			
Sterol	FS	SE	SG	ASG	FS	SE	SG	ASG
Cholesterol (**2**)	25[a]	56	3.5	89.5	15	81	36	48
Epibrassicasterol (**4**)	68	6	93.5	10.5	83	—	64	—
24-Methylenecholesterol (**5**)	—	—	—	—	—	10	—	28.5
(24ξ)-24-Methylcholest-5-en-3β-ol (**6**)	—	—	—	—	—	1	—	—
(24ξ)-24-Ethylcholesta-5,22*E*-dien-3β-ol (**7**)	7	38	—	—	1	3	—	—
(24ξ)-24-Ethylcholest-5-en-3β-ol (**8**)	—	trace	—	—	1	3	—	—
Isofucosterol (**9**)	—	trace	—	—	—	2	—	—
Unknown	—	—	3	—	—	—	—	23

[a]Percentage of total sterols in each sterol class. See Table 2 for abbreviations.

DISCUSSION

Evidence is presented here that in *P. tricornutum,* as well as in higher plants (19) and in other types of algae (20), sterols occur as free, ester, and glycosylated forms. Under standard culture conditions (i.e., at 13°C under white light), free sterols are not the major compounds since 64% of total sterols are glycosylated. The occurrence of both SG and AGS suggests that an UDPG-sterol:β-Dglucosyltranferase and an SG:acyltransferase are likely involved in the synthesis of these compounds as demonstrated in the chlorophyte *Prototheca zopfii* (21).

Both the sterol profile inside each class and the distribution of sterols between free and conjugated forms were shown here for the first time to be closely dependent on illumination and growth temperature conditions. The temperature shift from 13 to 23°C was found to trigger a dramatic decrease in free and conjugated sterol biosynthesis. The slowing down of the biosynthesis was attested by the relative accumulation of 24-methylcholesterol (**5**) in SE, resulting in a lower flux of free precursors toward the synthesis of epibrassicasterol (**4**). Whatever the light conditions, an important increase in the relative percentage of cholesterol was also found in all forms of sterols, especially in SE, with a parallel decrease in the amount of epibrassicasterol, indicating a significant inhibition of the first methylation step at C-24.

Sterol glycosylation appears to be very sensitive to environmental changes. Thus, this process was found to be stimulated in cells grown at 13°C under red and blue light, but strongly inhibited at 23°C (a 100-fold decrease in SG under blue light and a 100-fold decrease in ASG under red light). SE synthesis appears to be much less sensitive to environmental changes.

Under blue light, the photosystems of *P. tricornutum* are expected to have their greatest efficiency in regard to carbon fixation and high amounts of chlorophylls *a* and *c* (22), in agreement with the life conditions of this temperate diatom in the euphotic zone of the ocean where blue light prevails. Such culture conditions under nonstressing light illumination could be used in order to study the physiological significance of drastic changes on sterol composition (i.e., shift from SG to FS and dominance of cholesteryl glycosides instead of epibrassicasteryl glycosides at 23°C), in particular their effect on properties of *P. tricornutum* membranes.

Although contents and proportions of algal fatty acids are considered the most important aspects of lipids in animal nutrition, dietary requirements for sterols in Bivalves and other marine invertebrates should be more closely investigated. Op-

TABLE 4
Relative Sterol Composition of Free and Conjugated Forms of Sterols in *Phaeodactylum tricornutum* Grown at 13 and 23°C Under Green Light

	Growth temperature							
	13°C				23°C			
Sterol	FS	SE	SG	ASG	FS	SE	SG	ASG
22-Dehydrocholesterol (**1**)	2[a]	43	—	1.5	—	—	—	—
Cholesterol (**2**)	2	8	1	1.5	57.5	52.5	10	62.5
Cholestanol (**3**)	—	—	—	—	—	trace	—	—
Epibrassicasterol (**4**)	93	3.5	98.5	96	42.5	trace	86	30.5
24-Methylenecholesterol (**5**)	—	—	—	—	—	42	—	—
(24ξ)-24-Methylcholest-5-en-3β-ol (**6**)	0.5	—	—	—	—	—	—	—
(24ξ)-24-Ethylcholesta-5,22*E*-dien-3β-ol (**7**)	0.5	5	—	—	—	2.5	—	—
(24ξ)-24-Ethylcholest-5-en-3β-ol (**8**)	1	34	—	—	—	1.5	—	—
Isofucosterol (**9**)	1	6.5	—	—	—	1	—	—
Unknown	—	—	trace	1	—	—	4	7

[a]Percentage of total sterols in each sterol class. See Table 2 for abbreviations.

TABLE 5
Relative Sterol Composition of Free and Conjugated Forms of Sterols in *Phaeodactylum tricornutum* Grown at 13 and 23°C Under Yellow Light

	Growth temperature							
	13°C				23°C			
Sterol	FS	SE	SG	ASG	FS	SE	SG	ASG
22-Dehydrocholesterol (**1**)	4[a]	66.5	—	1.5	—	—	—	—
Cholesterol (**2**)	2	7.5	2.5	0.5	12	83.5	11	65.5
Cholestanol (**3**)	—	3.5	—	—	—	—	—	—
Epibrassicasterol (**4**)	91.5	3	97.5	96	82	1.5	29.5	3
24-Methylenecholesterol (**5**)	—	—	—	trace	—	6.5	1	5
(24ξ)-24-Methylcholest-5-en-3β-ol (**6**)	1	—	—	—	—	2.5	—	—
(24ξ)-24-Ethylcholesta-5,22*E*-dien-3β-ol (**7**)	0.5	19.5	—	—	4.5	1.5	—	—
(24ξ)-24-Ethylcholest-5-en-3β-ol (**8**)	1	—	—	—	1.5	2.5	—	—
Isofucosterol (**9**)	—	—	—	—	—	2	—	—
Unknown	—	—	—	2	—	—	58.5	26.5

[a]Percentage of total sterols in each sterol class. See Table 2 for abbreviations.

timal amounts of sterols for animal growth and survival remain to be determined, but we have proven here that algal sterol contents, such as those exemplified by *P. tricornutum*, can be strongly modified by environmental factors. Whether or not sterol conjugates can satisfy the sterol requirement as efficiently as free forms has to be questioned. More suitable algal diets could therefore be provided in hatcheries by manipulating culture conditions.

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TABLE 6
Relative Sterol Composition of Free and Conjugated Forms of Sterols in *Phaeodactylum tricornutum* Grown at 13 and 23°C Under White Light

	Growth temperature							
	13°C				23°C			
Sterol	FS	SE	SG	ASG	FS	SE	SG	ASG
Cholesterol (**2**)	3.5[a]	59	trace	13	21	77	12	20
Epibrassicasterol (**4**)	95	33	99	80	75	3.5	82	59
(24ξ)-24-Methylcholest-5-en-3β-ol (**6**)	0.5	—	trace	—	trace	4	—	—
(24ξ)-24-Ethylcholesta-5,22*E*-dien-3β-ol (**7**)	—	—	—	—	2	6.5	—	—
(24ξ)-24-Ethylcholest-5-en-3β-ol (**8**)	1	8	—	—	2	4	—	—
Isofucosterol (**9**)	—	—	—	—	—	5	—	—
Unknown	—	—	trace	7	—	—	6	21

[a]Percentage of total sterols in each sterol class. See Table 2 for abbreviations.

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A Simple Method for the Synthesis of Ceramides and Radiolabeled Analogues

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ABSTRACT: A simple method has been developed for the coupling of amines to carboxylic acids. *N*-Fatty-acyl-sphingosine, cerebroside, and GM_3, as well as their respective [^{14}C] analogues, were synthesized using diethylphosphoryl cyanide as a potent coupling agent in the presence of triethylamine. The reaction procedure is rapid, racemization-free, and utilizes acids without derivatization. The desired ceramide products were obtained in 85–90% yield within one hour. The facile method presented here can also be used to synthesize [^{3}H]-*N*-acyl-labeled, as well as [^{14}C] [^{3}H] double-labeled, ceramides. *Lipids 31*, 995–998 (1996).

Ceramide (*N*-fatty acyl sphingosine) is the lipid moiety of sphingomyelin and all types of glycosphingolipids. It is considered the structural unit which anchors sphingolipids to biological membranes. It is the precursor as well as degradation product of sphingomyelin and glycosphingolipids (1). Recently, the functional role of ceramide as second messenger in transmembrane signaling has been the subject of great interest. When cells are stimulated by TNF-α, IL-1β, γ-interferon, dexamethasone, etc., ceramide content increases because of increased sphingomyelinase level and, consequently, activates ceramide-dependent kinases. This event leads to various phenotype changes, possibly through activation of transcription factors (see Refs. 2,3 for review). Ceramide can also be produced by hydrolysis of glucosyl- or galactosylceramide by β-glucosidase or β-galactosidase, as well as by endoceramidase (4,5).

The knowledge of the regulatory mechanisms for ceramide synthesis as a part of sphingolipid metabolism, and its degradation into sphingosine and fatty acid, is increasingly important. Thus the availability of [^{14}C]-labeled ceramide analogue, or preferably [^{14}C][^{3}H] double-labeled analogue, is highly desirable.

In order to study the effect of exogenous ceramides in cell growth and differentiation and to quantitate the ceramidase assay, we synthesized a variety of unlabeled, [^{14}C]-labeled, and [^{14}C][^{3}H] double-labeled ceramide homologues **3**, **5**, **8**, **10**, and **11** by a simple and convenient method.

Amides and ceramides are normally prepared using the acid anhydride (6,7), acid-halides (8,9) or by transacylation *via* fatty acid *N*-hydroxy succinimide (10,11) or by *p*-nitrophenyl esters (12,13). Alternative methods, using coupling agents as *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (14), dicyclohexylcarbodimide (15), or diphenyl phosphorazidate (16), have also been applied, but most of these reagents either require the removal of precipitated salts or result in unwanted side-products. These side-products adhere to the desired ceramides and cause interference in the biological assays. Some of these coupling agents have the tendency to inactivate at room temperature and some produce the undesired acid anhydrides, due to a rapid breakdown of the transition intermediate (17). Most of the reported procedures for the preparation of radiolabeled derivatives use the radioactive acids (acid derivatives) in large excess amount, as compared to amines, and thereby result in unnecessarily huge amounts of radioactive waste. In the procedure reported here, we used stoichiometeric amounts of the labeled acids, and we obtained the coupled products in almost quantitative yields.

MATERIALS AND METHODS

Sphingosine, **1**, was synthesized by the method of Herold (18). 1-[^{3}H]-Sphingosine, **4**, was generously supplied by Toyokuni *et al.* (19). Psychosine, **6**, was obtained by the alkaline hydrolysis of galactocerebroside isolated from bovine brain (20,21). *O*-(2-Methyl-5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-d-glycero-α-D-galacto-2-nonulopyranosylonate)-(2-3)-*O*-(2,4-di-*O*-acetyl-6-*O*-benzoyl-β-D-galactopyranosyl)-(1-4)-*O*-(3-*O*-acetyl-2,6-di-*O*-benzoyl-β-D-glucopyranosyl)-(1-1)-2S3R, 4E-3-*O*-pivolyl-2-amino-4-octadecene-1,3-diol (derivatised lyso-GM_3), **9**, was synthesized by the modified method of Murase *et al.* (22). Diethylphosphoryl cyanide (DEPC) was purchased from Aldrich Chemicals (Milwaukee, WI), sodium octonate [^{14}C] and [^{14}C] palmitic acid were purchased from ICN Chemicals (Cleveland, OH).

The ^{1}H nuclear magnetic resonance spectra were recorded on a Bruker (Karlsruhe, Germany) WM-500 spectrometer in

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Abbreviation: DEPC, diethylphosphoryl cyanide.

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$CDCl_3$, $CDCl_3/CD_3OD$ (1:1), or dimethyl sulphoxide/D_2O (98:2). Thin-layer chromatography was performed on precoated silica gel plates (visualized by spraying with orcinol in H_2SO_4). Column chromatography was performed on silica gel. Fast atom bombardment–mass spectrometry was recorded on JEOL (Tokyo, Japan) JMS-10HX 110/DA-5000 mass spectrometer. Radioactivity was measured on a Beckman (Palo Alto, CA) LS 3801 liquid scintillation counter.

A representative procedure is as follows: Triethylamine (2.5 micromole) was added at 0°C to a stirred mixture of carboxylic acid (3 micromol), DEPC (6 micromole), and sphingoamine derivative (3 micromole) in a mixture of dimethylformamide and dichloromethane (2 mL, 1:3). The reaction mixture was stirred for 45 min at 0–30°C under argon, and progress of reaction was monitored by thin-layer chromatography with $CHCl_3/CH_3OH$ (18:2) solvent mixture. After the completion of reaction (45–60 min), the reaction mixture was evaporated under reduced pressure and the residue was subjected to column chromatography to isolate the desired ceramide derivatives with haxane, hexane/$CHCl_3$ (1:1), and $CHCl_3/CH_3OH$ (99:1).

Ceramides and cerebrosides: *N*-octanoyl-sphingosine **3a**, **3b**, and **5b** and *N*-palmitylpsychosine **8a** and **8b** were prepared, from sphingosine **1**, 1-[^{3}H]-sphingosine **4**, and psychosine **6**, respectively. *N*-Palmityl-GM_3 derivatives **10a** and **10b** were synthesized from the protected lyso-GM_3 **9** and later transformed into the unprotected derivatives **11a** and **11b** by treatment with $NaOCH_3$/NaOH.

Synthesis of all these ceramide derivatives was complete within one hour, as indicated by thin-layer chromatographic analysis of reaction mixtures. Prolongation of reaction time (up to 72 h) did not exhibit any appreciable increase in the yield of reaction.

The ^{1}H nuclear magnetic resonance and mass values of ceramides **3**, **5**, **8**, **10**, and **11**, were found to be in good agreement with the published data (Fig. 1).

Authentic samples of ceramides, *N*-octanoyl-sphingosine, psychosine, and GM_3 were compared with **3a**, **3b**, **5b**, **8a**, **8b**, **11a**, and **11b**, and found identical by thin-layer chromatographic co-spotting (Fig. 2), in addition to spectral data. The labeled ceramides **3b**, **5b**, **8b**, **10b**, and **11b** exhibited radioactive purity of 92–98%.

RESULTS AND DISCUSSION

We have synthesized ceramides (see Scheme 1): 2S, 3R, 4E-2-(*N*-octanoylamino-octadecene-1,3-diol (*N*-octanoyl-sphingosine), **3a**; 2S, 3R, 4E-1-*O*-(β-D-galactopyranosyl)-2-hexadecanoylamino-octadecene-1,3-diol [*N*-palmityl psychosine (cerebroside)], **8a**; and *O*-(5-*N*-acetylamino-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2-3)-*O*-(β-D-galactopyranosyl)-(1-4)-*O*-(β-D-glucopyranosyl)-(1-1)-2S, 3R, 4E-2-(*N*-hexadecanoylamino-4-octadecene-1,3-diol (*N*-palmityl GM_3), **11a**, as well as their [^{14}C]-labeled analogues, **3b**, **8b**, and **11b**, respectively. A double-labeled ceramide: *N*-[^{14}C]-octanoyl-1-[^{3}H]-sphingosine, **5b**, was also synthesized.

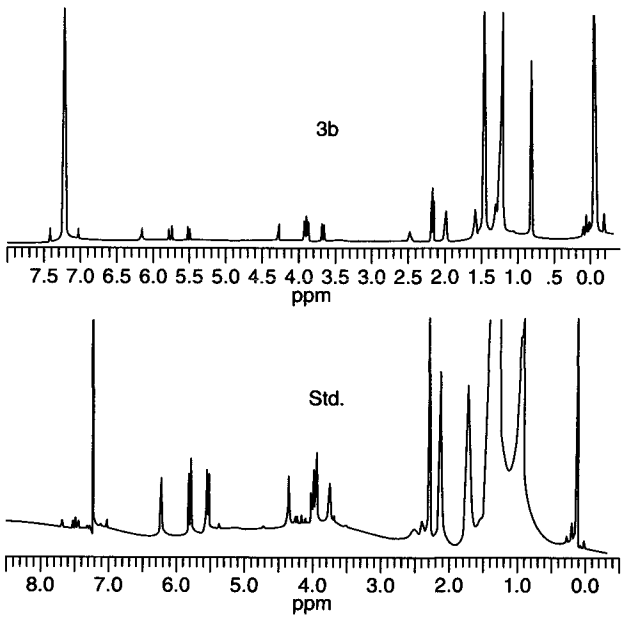


FIG. 1. The ^{1}H nuclear magnetic resonance spectrum of *N*-octanoyl-sphingosine **3b** and **std.** recorded in $CDCl_3$.

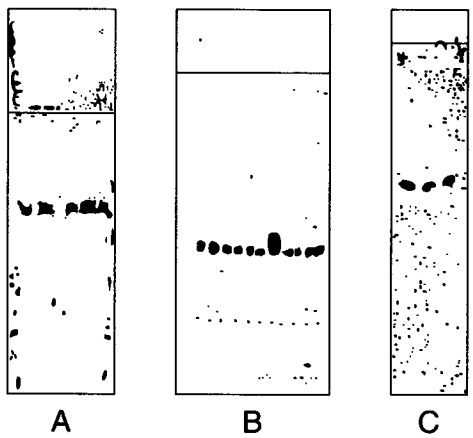
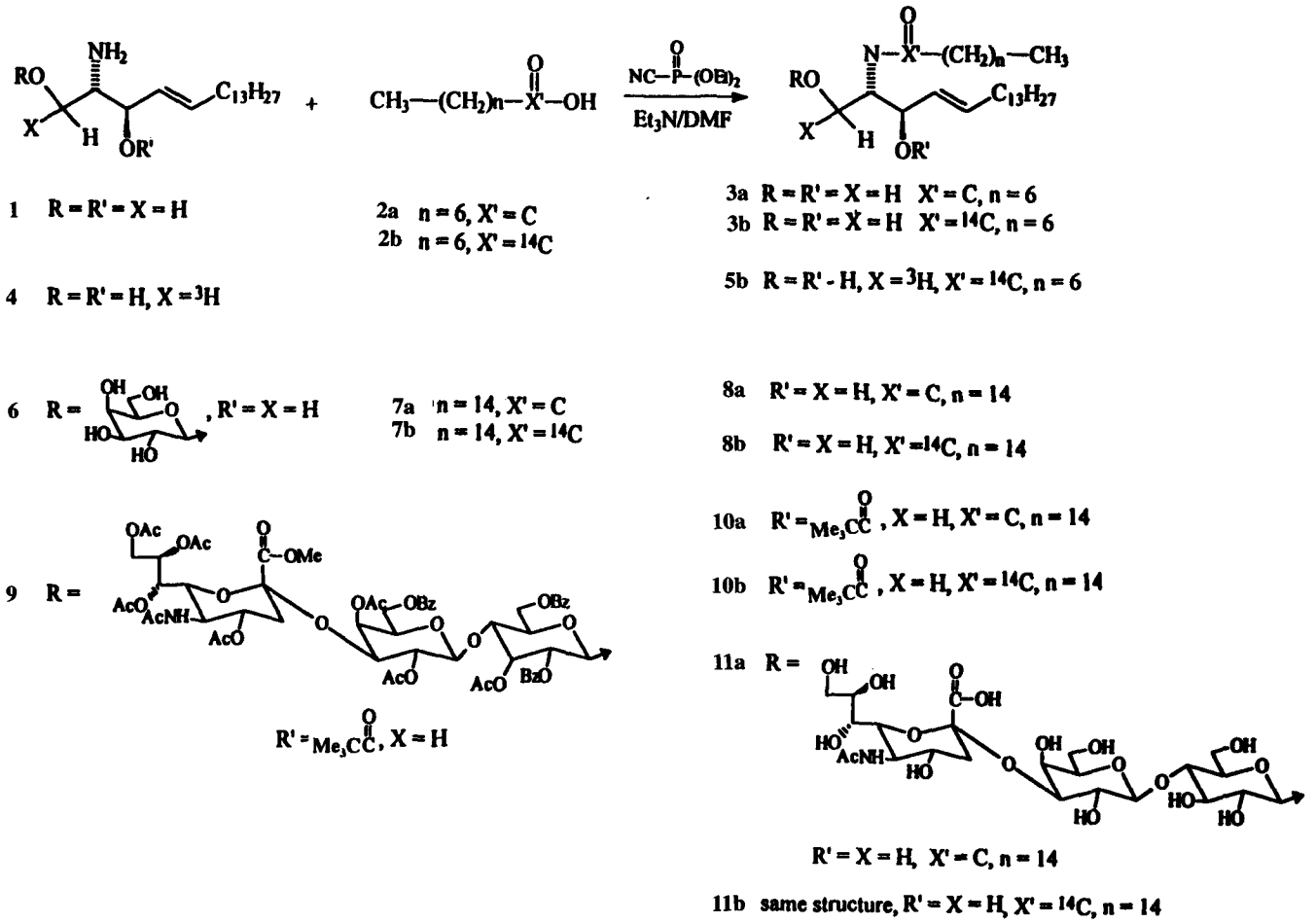


FIG. 2. Thin-layer chromatograms comparing the authentic samples with respective ceramide analogues. A : *N*-Octanoyl-sphingosine **3b**, **std.** and **5b** [$CHCl_3$:CH_3OH (18:2)]; B: *N*-Palmityl-psychosine **8b** and **std.** [$CHCl_3$:CH_3OH (17:3)] and C: *N*-Palmityl-GM_3 **11b** and **std.** [$CHCl_3$:CH_3OH (18:2)].

All of the above ceramides were obtained in 85–90% overall yields, using a facile synthetic procedure in which DEPC was used as a coupling agent. DEPC was first introduced by Yamada *et al.* (23,24) in 1973 as an efficient coupling agent for peptide synthesis. In their report the group also specified that the cyano intermediate formed after the action of DEPC can react with hydroxyl group, if left unprotected, to produce an ester derivative of the acid. However, during our synthetic studies, when we reacted different sphingoamines containing unprotected hydroxyl group with various carboxylic acids and



SCHEME 1

their [^{14}C] analogues, we obtained exclusively the desired ceramide products. Also, we did not obtain any esterified side-products due to the fatty acid and free hydroxyl group coupling.

The simple method described here is rapid, convenient, and 95% of the reaction takes place within the first 40 min, producing the desired ceramides with complete retention of configuration. During our isolation step, we have eliminated the aqueous work-up of the reaction mixture and thus reduced the extent of radioactive waste and exposure.

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ANNOUNCEMENTS

CALENDAR

October 1996

Frontiers in Lipid and Lipoprotein Research: Basic Science, Analytical, Clinical, and Public Health Applications, Oct. 4–6, 1996, Stouffer Renaissance Hotel, Dallas, Texas. Sponsored by the Lipids and Lipoproteins Division of the American Association for Clinical Chemistry, the Centers for Disease Control and Prevention, and the American Heart Association. An integrated review of relevant recent advances in basic science, measurement technology, epidemiological and clinical research, and public health issues will be presented. This review will help define state-of-the-art techniques for understanding and preventing morbidity and mortality due to heart disease. Information: Paula M. Steiner, Medical Research Laboratories, 2 Tesseneer Dr., Highland Heights, KY 41076 (Phone: 606-781-8877, Extension 252; Fax: 606-781-9310; E-mail: lipf@cehehll.em.cdc.gov).

American College of Nutrition, 37th Annual Meeting, Oct. 11–13, 1996, San Francisco, California (Phone: 212-777-1037; Fax: 212-777-1103).

Certification Board for Nutrition Specialists, Oct. 11, 1996, San Francisco, California. Certifying examination for nutritionists and dieticians with advanced degrees. Information: CBNS, 1562 First Avenue, Box #258, New York, NY 10028 (Phone: 212-332-0774 (leave message); Fax: 212-777-1103).

Seminar: Interconversion of Polyunsaturated Fatty Acids, and Their Functions in Membranes, Oslo, Norway, Oct. 11–12, 1996. Information: Georg Lambertsen, Secretary, Lipidforum, Breistølen 1, N-5035, Bergen, Norway (Phone: 4755 + 32 89 56; Fax: 47 55 + 32 57 24).

"Confectionery Fats" Symposium, Belgrave Sq., London, Oct. 15–16, 1996, Oils and Fats Group—Society of the Chemical Industry, UK. Information: Secretariat, SCI, 14/15 Belgrave Sq., London.

November 1996

AOCS Conference on PUFA in Infant Nutrition: Consensus and Controversies, Nov. 7–9, 1996, Barcelona, Spain. Information: Susan E. Carlson (E-mail: scarlson@utmem1.utmem.edu); Robert A. Gibson (E-mail: rgibson@paed_nnu.fmc.flinders.edu.au); Berthold Koletzko (Fax: 49-89-5160-4733); or AOCS Meetings Department, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

March 1997

CERLIB 3rd Winter Research Conferences, Les Arcs 1800, France, March 9–14, 1997. Proteins and Free Radicals, from Radical Enzymes to Damages; Oxidative Effects of Radiations on Biomolecules and Cells; Oxidative Stress and Apoptosis. Information: Arlette Alcaraz, Laboratoire de Biochimie C, CHU Grenoble BP 217, 38043 Grenoble Cédex 9, France (Phone: 33 76 76 57 54; Fax: 33 76 76 56 64; E-mail: CERLIB@ujf-grenoble.fr).

April 1997

IV. International Dermatology Symposion Berlin, Sebaceous Gland and Its Disorders, Basic and Clinical Research, Clinical Entities and Treatment, April 11–13, 1997, University Medical Center Benjamin Franklin. Information: Docent Dr. Ch. C. Zouboulis, Department of Dermatology, University Medical Center Benjamin Franklin, The Free University of Berlin, Hindenburgdamm 20, D-12200 Berlin, Germany (Phone: 49-30-8445-2808; Fax: 49-30-8445-4262).

June 1997

The 19th Nordic Lipid Symposium, Ronneby, Sweden, June 15–18, 1997. Information: Georg Lambertsen, Secretary, Lipidforum, Breistølen 1, N-5035, Bergen, Norway (Phone: 47 55 + 32 89 56; Fax: 47 55 + 32 57 24).

July 1997

Fourth International Congress on Essential Fatty Acids and Eicosanoids, July 20–24, 1997, Edinburgh International Conference Centre, Scotland, United Kingdom. Contact: Vicki Grant/Wendy Adesegun, c/o Meeting Makers, 50 George St., Glasgow G1 1QE, Scotland, United Kingdom [Phone: 44 (0) 141 553 1930; Fax: 44 (0) 141 552 0511; E-mail: mm@meetingmakers.co.uk].

Bisallylic Hydroxylation and Epoxidation of Polyunsaturated Fatty Acids by Cytochrome P450

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ABSTRACT: Polyunsaturated fatty acids can be oxygenated by cytochrome P450 to hydroxy and epoxy fatty acids. Two major classes of hydroxy fatty acids are formed by hydroxylation of the ω-side chain and by hydroxylation of bisallylic methylene carbons. Bisallylic cytochrome P450-hydroxylases transform linoleic acid to 11-hydroxylinoleic acid, arachidonic acid to 13-hydroxyeicosa-5*Z*,8*Z*,11*Z*,14*Z*-tetraenoic acid, 10-hydroxyeicosa-5*Z*,8*Z*,11*Z*,14*Z*-tetraenoic acid and 7-hydroxyeicosa-5*Z*,8*Z*,11*Z*,14*Z*-tetraenoic acid and eicosapentaenoic acid to 16-hydroxyeicosa-5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-pentaenoic acid, 13-hydroxyeicosa-5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-pentaenoic acid and 10-hydroxyeicosa-5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-pentaenoic acid as major metabolites. The bisallylic hydroxy fatty acids are chemically unstable and decompose rapidly to *cis-trans* conjugated hydroxy fatty acids during acidic extractive isolation. Bisallylic hydroxylase activity appears to be augmented in microsomes induced by the synthetic glucocorticoid dexamethasone and by some other agents, but the P450 gene families of these hydroxylases have yet to be determined. The fatty acid epoxides, which are formed by cytochrome P450, are chemically stable, but are hydrolyzed to diols by soluble epoxide hydrolases. Epoxidation of polyunsaturated fatty acids is a prominent pathway of metabolism in the liver and the renal cortex and epoxygenase activity appears to be under homeostatic control in the kidney. Many arachidonate epoxygenases have been identified belonging to the CYP2C gene subfamily. Epoxygenases have also been found in the central nervous system, endocrine organs, the heart and endothelial cells. Epoxides of arachidonic acid have been found to exert pharmacological effects on many cells.

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Abbreviations: CYP, cytochrome P450; EDHF, endothelium-derived hyperpolarization factor; EGF, epidermal growth factor; GC–MS, gas chromatography–mass spectrometry;16-HEPE, 16-hydroxyeicosa-5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-pentaenoic acid; 13-HEPE, 13-hydroxyeicosa-5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-pentaenoic acid; 10-HEPE, 10-hydroxyeicosa-5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-pentaenoic acid; HETE, hydroxyeicosatetraenoic acid; 7-HETE, 7-hydroxyeicosa-5*Z*,8*Z*,11*Z*,14*Z*-tetraenoic acid; 10-HETE, 10-hydroxyeicosa-5*Z*,8*Z*,11*Z*,14*Z*-tetraenoic acid; 13-HETE, 13-hydroxyeicosa-5*Z*,8*Z*,11*Z*,14*Z*-tetraenoic acid; HODE, hydroxyoctadecadienoic acid; 8-HODE, 8-hydroxyoctadeca-9*Z*,12*Z*-dienoic acid; 9-HODE, 9-hydroxyoctadeca-10*E*,12*Z*-dienoic acid; 11-HODE, hydroxyocta-9*Z*,12*Z*-deca-9*Z*,12*Z*-dienoic acid; 13-HODE, 13-hydroxyoctadeca-9*Z*,11*E*-dienoic acid; 14-HODE, 14-hydroxyoctadeca-9*Z*,12*Z*-dienoic acid; 16-HODE, 16-hydroxyoctadeca-9*Z*,12*Z*-dienoic acid; HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; LTA_4, leukotriene A_4; P450, cytochrome P-450; PGH, prostaglandin H; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acids; TAO, troleandomycin.

Cytochrome P450 (P450) designates a group of heme-thiolate proteins, which catalyze oxygenation of xenobiotics and endogenous compounds, e.g., fatty acids, lipid hydroperoxides, steroids, bile acids, retinoids, prostaglandins, leukotrienes, and cytokines (1–3). The mammalian P450 enzymes are formed from a gene superfamily presently comprising 14 gene families and 26 subfamilies (2,4). Over 400 P450 have been cloned and sequenced and about 200 of them are mammalian (e.g., 35 human, 48 rat, 29 mice, and 28 rabbit P450; Ref. 4).

According to recent work, the P450 gene superfamily has a long history of evolution (2). The ancestral gene may have existed 3.5 billion years ago. Many P450 can be induced by xenobiotics, often as part of a detoxification process, and the induction of P450 has attracted attention as an example of gene regulation by drugs (5). The evolution of P450 suggests that P450 gene regulation might have evolved as a metabolic regulatory pathway for endogenous compounds and among them, fatty acids.

The atomic, three-dimensional structure of a mammalian P450 has not yet been determined. P450 is also present in plants, fungi, and bacteria. The fatty acid monooxygenase P450BM-3 of *Bacillus megaterium*, P450cam of *Pseudomonas putida,* P450terp of a *Pseudomonas* species, and P450eryF of *Saccharopolyspora erythrea* have been crystallized and have provided three-dimensional structural and catalytic information on P450 (6–10,11). Reviews on the P450 gene superfamily with update on new sequences, gene mapping, and recommended nomenclature are published regularly (see Ref. 2), and recent information can also be conveniently obtained from the Internet (4,11).

OXYGENATION OF POLYUNSATURATED FATTY ACIDS

Arachidonic acid, linoleic acid, and other polyunsaturated fatty acids (PUFA) can be oxygenated by P450 in several

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ways: (i) hydroxylations of the ω-side chain (ω1, ω2, ω3, etc.); (ii) hydroxylation of bisallylic carbons; (iii) epoxidation; (iv) hydroxylation of allylic carbons; (v) hydroxylation with double-bond migration.

The first three oxygenation mechanisms are quantitatively predominant. The oxygenation of arachidonic acid by bisallylic P450-hydroxylases and P450-epoxygenases is summarized in Figure 1. Many other PUFA can be oxygenated in the same way. Arachidonic acid has been intensively investigated due to its biological importance as a precursor of autacoids in mammals. Linoleic acid seems to have a corresponding function in lower organisms, algae and fungi, and in plants. Linoleic acid is also a major unsaturated fatty acid in the human diet. The present review will focus on bisallylic hydroxylations and epoxidations of PUFA, and in particular on linoleic, arachidonic, and eicosapentaenoic acids. As regards nomenclature, the recommendations on abbreviations (12) will be followed. Epoxyeicosatrienoic acid and the corresponding dihydroxyeicosatrienoic acid are often, and incorrectly, abbreviated to EET and to DHET by many investigators. We will use the abbreviations EpETrE and DiHETrE according to the standard nomenclature only to avoid confusion with other epoxides and diols of unsaturated eicosanoic fatty acids. We do so with some reluctance. EET is a widely recognized and obviously a more convenient abbreviation than EpETrE.

BISALLYLIC HYDROXYLATIONS

Early studies clearly demonstrated that arachidonic acid was converted enzymatically, and not by lipid peroxidation, to six *cis-trans* conjugated hydroxyeicosatetraenoic acids (HETE) by rat liver microsomes and NADPH (13–15). Steric analysis showed that five of the six *cis-trans* conjugated HETE were racemic or nearly racemic mixtures. One compound, 12*R*-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid (12*R*-HETE), was formed with stereoselectivity (81%) (16). The mechanism of biosynthesis was not determined. A hypothetical pathway for biosynthesis of 12*R*-HETE from 11,12-epoxy-5,8,14-eicosatrieonic acid (11,12-EpETrE) was proposed (see Ref. 17 for review). Many aspects of the biosynthesis of HETE by P450 remained conjectural, and it was unknown whether these HETE contained oxygen from air or water.

It is well established that the mechanism of ω2-hydroxylation of fatty acids by P450 involves hydrogen abstraction and insertion of the hydroxyl group with retention of configuration (18). More recently, the ω-hydroxylation of (1*R*)- and (1*S*)-[1-^{3}H, ^{2}H, ^{1}H;1-^{14}C]octane to 1-octanol by microsomes of rat liver and NADPH was investigated by Shapiro *et al.* (19). They found that the incoming hydroxyl assumed the configuration of the displaced hydrogen. However, there are exceptions to this rule. The incoming hydroxyl may also assume the opposite configuration (20). For this and other reasons, it

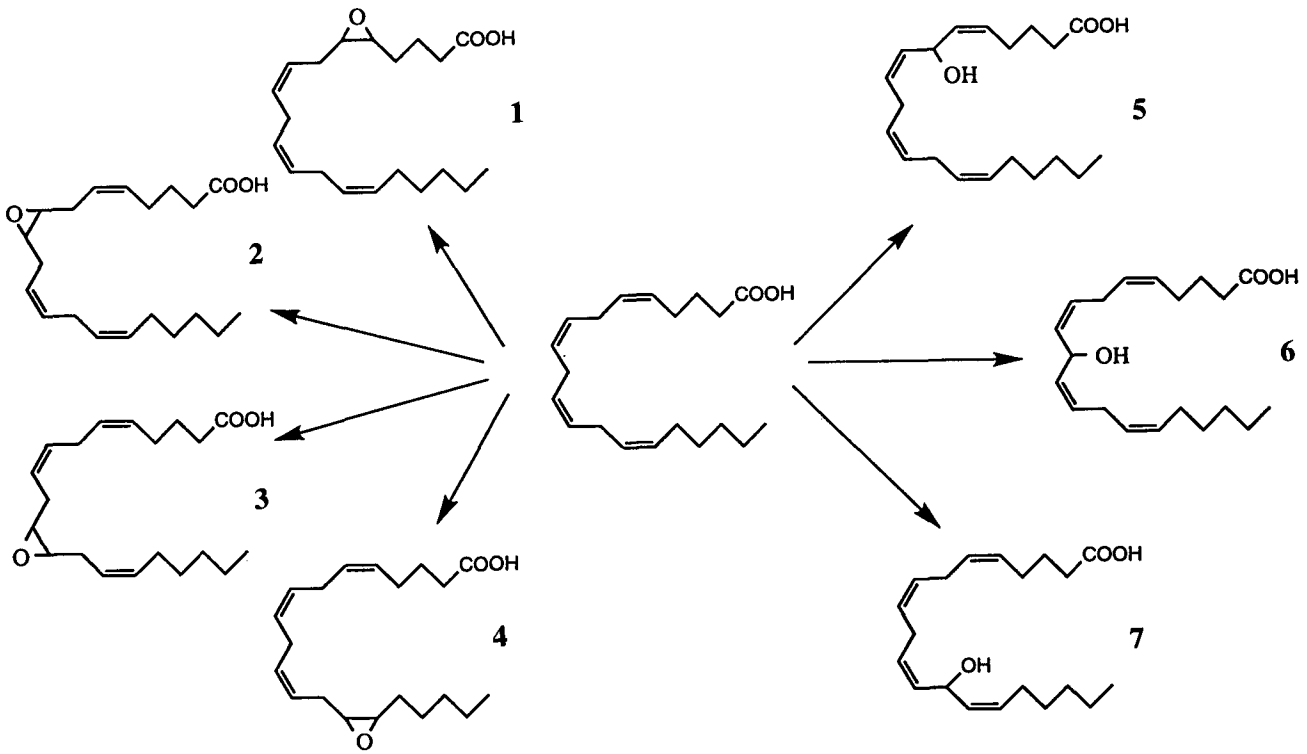


FIG. 1. Epoxidation and bisallylic hydroxylation of arachidonic acid by hepatic monooxygenases. The structures marked 1, 2, 3, and 4 designate the 5,6-, 8,9-, 11,12-, and 14,15-epoxides of arachidonic acid, respectively, while 5, 6, and 7 designate 7-hydroxyeicosa-5*Z*,8*Z*,11*Z*,14*Z*-tetraenoic acid (HETE), 10-HETE and 13-HETE, respectively.

seemed of interest to determine the mechanism of hydrogen abstraction and oxygenation during P450-catalyzed biosynthesis of *cis-trans* conjugated HETE. For simplicity, linoleic acid was chosen as a model substrate. The results were then extended to arachidonic and eicosapentaenoic acids.

Linoleic acid was metabolized by liver microsomes from untreated or phenobarbital-treated rats in the presence of NADPH to a large number of metabolites (21). Many metabolites had been previously identified as products formed by epoxidation and ω side-chain hydroxylations (22–24). Novel metabolites were 8-hydroxy-9,12-octadecadienoic acid (8-HODE), 11-HODE, and 14-HODE. In addition, two *cis-trans* conjugated hydroxy fatty acids, 9-HODE and 13-HODE, were detected. 11-HODE (59% *R*) was found to be a major metabolite, provided that the metabolites were extracted at pH 5–6 (21). Under these conditions, only small amounts of 13-HODE and 9-HODE were formed. Steric analysis revealed that 13-HODE and 9-HODE consisted mainly of the *R* enantiomers (>80%), while 11-HODE was almost racemic.

11-HODE was first identified by Hamberg *et al.* (25) in a red alga, *Lithothamnion corallioides*. These investigators found that 11-HODE was unstable in acidic media and converted to equal amounts of racemic 9-HODE and racemic 13-HODE (25). The latter contained oxygen from water. To avoid decomposition, the pH during extractive isolation of 11-HODE should be neutral or above pH 5.

Experiments under $^{18}O_2$ gas showed that 11-HODE was formed in liver microsomes by incorporation of oxygen from air (21). 13*R*-HODE and 9*R*-HODE also contained oxygen from air. The mechanism of biosynthesis of 9-, 11-, and 13-HODE was studied with stereospecifically deuterated linoleic acids. When [11*R*-^{2}H]linoleic and [11*S*-^{2}H]linoleic acids were used as substrates, the analysis showed a suprafacial relationship between oxygen insertion and hydrogen abstraction at C_{11} of the three enzymatic products, 11*R,S*-HODE, 9*R*-HODE, and 13*R*-HODE. A mechanism for the biosynthesis of these metabolites is proposed in Figure 2. 11-HODE was formed by hydroxylation of C_{11}, a process designated bisallylic hydroxylation. The two minor products, 8-HODE and 14-HODE, were formed by allylic hydroxylation, i.e., by hydroxylations of the allylic carbons C_8 and C_{14}, respectively. The experiments with stereospecifically deuterated linoleic acid indicated that 9*R*-HODE and 13*R*-HODE were formed by hydrogen abstraction at C_{11}, migration of the double bond, and suprafacial oxygen insertion at C_9 or C_{13} (Fig. 2). This sequence was designated hydroxylation with double-bond migration. To abstract hydrogen from the bisallylic methylene carbon requires about 10 kcal/mol less energy than abstraction of an allylic hydrogen and considerably less energy than abstraction of a methyl hydrogen (26).

In analogy with biosynthesis of 11-HODE from linoleic acid, arachidonic acid was found to be hydroxylated at C_{13},

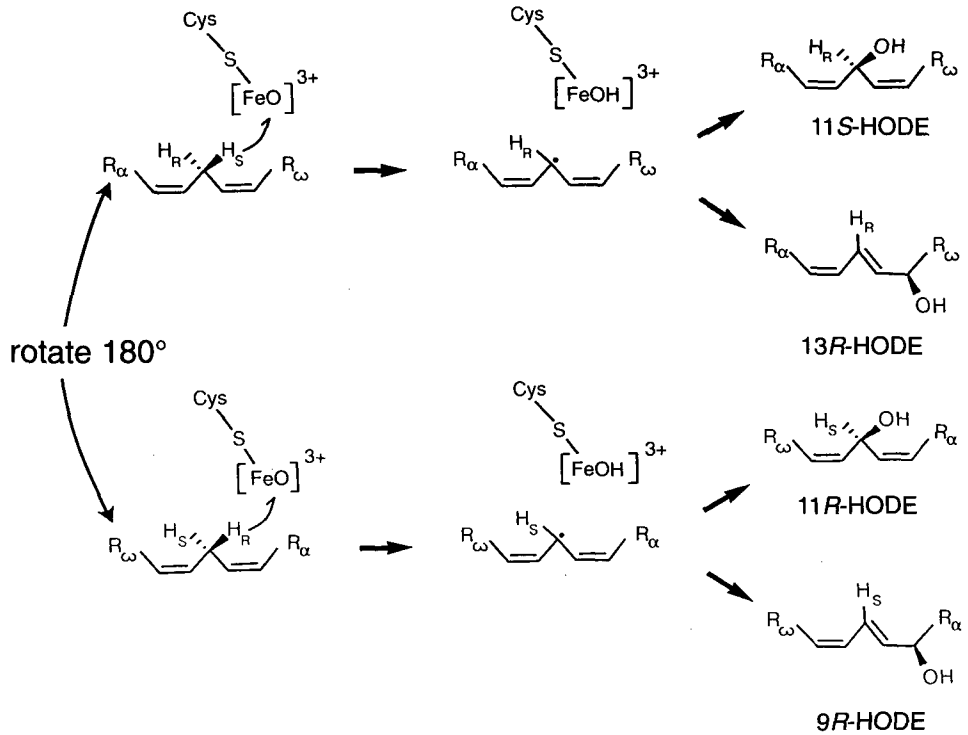


FIG. 2. A mechanism for the biosynthesis of 11-hydroxyoctadecadienoic acid (HODE), 13*R*-HODE, and 9*R*-HODE from linoleic acid. The substrate is rotated 180 degrees in the lower sequence. Top, biosyntheis of 11*S*-HODE and 13*R*-HODE *via* abstraction of the pro-*S* hydrogen. Bottom, biosynthesis of 11*R*-HODE and 9*R*-HODE *via* abstraction of the pro-*R* hydrogen. R_ω and R_α designate the terminal side chain and the carboxyl side chain, respectively.

the ω8 carbon, by monkey and human hepatic P450 to 13-HETE (27). Many other hydroxy metabolites were formed including 12*R*-HETE. The mechanism of biosynthesis of 12*R*-HETE is still enigmatic. It seems possible that 12*R*-HETE might be formed by hydrogen abstraction at C_{10}, double-bond migration and oxygen insertion at C_{12} in analogy with the biosynthesis of 13*R*-HODE from linoleic acid. This hypothesis should be tested with stereospecifically labeled arachidonic acid in position C_{10} and also with experiments under $^{18}O_2$ gas.

Brash *et al.* (28) investigated arachidonic acid metabolism by bisallylic hydroxylases of liver microsomes of phenobarbital-treated rats. Two novel metabolites were identified, 7-HETE and 10-HETE, as well as 13-HETE. The results showed that bisallylic hydroxylases may oxygenate all three bisallylic methylene carbons of arachidonic acid without positional selectivity. As described below, the major form of phenobarbital-induced P450 (CYP2B1) will epoxidize all four double bonds of arachidonic acid, while other epoxygenases show selectivity. Brash *et al.* (28) also made the unexpected observation that the bisallylic hydroxyl oxygen may, under certain conditions, be retained in the *cis-trans* conjugated products formed by acidic catalyzed decomposition of 13-HETE, 10-HETE, and 7-HETE.

11-HODE from linoleic acid and 13-HETE, 10-HETE, and 7-HETE from arachidonic acid were prominent metabolites of hepatic microsomes of phenobarbital-treated rats. 11-HODE and 18-HODE were formed in about equal amounts (21). The amounts of 13-HETE, 10-HETE, 7-HETE, 19-HETE, and 20-HETE were also of the same order of magnitude (28).

The bisallylic P450-hydroxylases have not been characterized with respect to their P450 gene families or subfamilies. Five different purified rat P450, i.e., CYP1A1, CYP2B1, CYP2B4, CYP2E1, and CYP4A1, and one rabbit P450 (CYP3A6) were investigated in a reconstituted system for bisallylic hydroxylase activity, but none of these formed significant amounts of [^{14}C]11-HODE from [^{14}C]linoleic acid (27). The negative results may partly be due to difficulties with reconstitution of the CYP3A subfamily (29).

Another approach was to study bisallylic hydroxylase activity by liver microsomes of treated rats. Drugs from five different classes of inducers of P450 were investigated (30), viz., β-naphthoflavone as an aromatic hydrocarbon-inducer (e.g., CYP1A), clofibrate as a peroxisome proliferator-inducer (CYP4A), the synthetic glucocorticoid dexamethasone as a steroid-inducer (CYP3A), phenobarbital as a prototype inducer (CYP2B), and acetone-starvation and isopropanol for CYP2E1. These agents may also induce many other P450 (5). CYP3A1 is also induced by phenobarbital and by ethanol or isopentanol (31,32).

All liver microsomes formed 18-HODE as a major metabolite while 11-HODE and 17-HODE were present in variable amounts. Liver microsomes of β-naphthoflavone-treated rats hydroxylated [^{14}C]linoleic acid at carbons 8, 11, 14, 16, 17, and 18. In addition, 9-HODE and 13-HODE were also formed. Microsomes of phenobarbital-treated rats formed essentially the same metabolites (21,28,30). 11-HODE was apparently formed without selectivity by liver microsomes of β-naphthoflavone and phenobarbital-treated rats. Liver microsomes of rats treated with acetone plus starvation or isopropanol also metabolized linoleic acid to 11-HODE along with large quantities of other hydroxy metabolites. These agents induce CYP2E1, which does not show bisallylic hydroxylase activity, as well as other P450, e.g., CYP3A1 (31). Clofibrate, which is a well-known inducer of ω1 hydroxylation (32,33), did not augment the biosynthesis of 11-HODE.

Liver microsomes of dexamethasone-treated Spague-Dawley and Fischer rats showed an augmented bisallylic hydroxylase activity (30). The two main metabolites of linoleic acid were 11-HODE and 18-HODE. Only small amounts of other hydroxy fatty acids were formed. Reverse phase–high-performance liquid chromatography (HPLC) analysis showed that dexamethasone treatment consistently increased formation of 11-HODE about tenfold in comparison with control rats (Fig. 3). Troleandomycin, an inhibitor of CYP3A (34), reduced the biosynthesis of 11-HODE (Fig. 3).

Bisallylic hydroxylations of arachidonic and eicosapentaenoic acids were also investigated (30). Microsomes of dexamethasone-treated rats formed mainly 13-HETE, 7-HETE, and 10-HETE, and only small amounts of 20-HETE and 19-HETE from [^{14}C]20:4n-6, while control microsomes formed 20-HETE, 13-HETE, and 19-HETE as the three major products. Microsomes of dexamethasone-treated rats converted [^{14}C]20:5n-3 to 13-hydroxyeicosapentaenoic acid (13-HEPE), 16-HEPE, and 10-HEPE as major bisallylic hydroxy metabolites and to 20-HEPE and 19-HEPE.

These results suggest that bisallylic hydroxylases for linoleic, arachidonic, and eicosapentaenoic acids could be induced by dexamethasone treatment of Sprague-Dawley and Fischer rats. Whether the bisallylic hydroxylases mainly belong to the CYP3A subfamily will await future studies with purified enzymes or expressed cDNA.

Bisallylic hydroxylases may be present in other gene families. Rifkind *et al.* (35) reported that five out of ten cDNA-

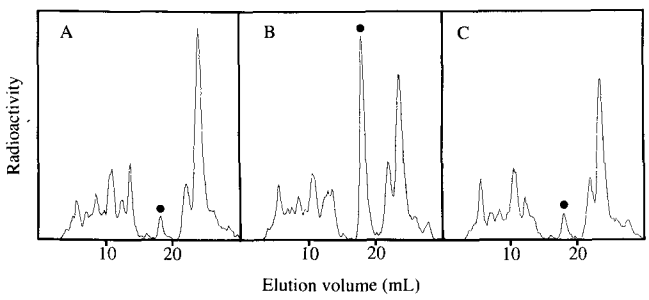


FIG. 3. Reverse-phase high-performance liquid chromatography of products formed from male rat liver microsomes incubated with NADPH and radiolabeled linoleic acid. A, control microsomes. B, microsomes from dexamethasone-treated rats. C, microsomes from dexamethasone-treated rats incubated in the presence of 10 μM troleandomycin. The filled circle marks 11-HODE. See Figure 2 for abbreviations.

expressed human P450 in HepG2 cells converted arachidonic acid to significant amounts of *cis-trans* conjugated HETE (CYP2C9 20% of total metabolites; CYP1A2 15%; CYP2C8 6%; CYP2B6 6%; CYP3A4, 5%). Whether these HETE were formed enzymically, by lipid peroxidation or from acid-catalyzed decomposition of bisallylic hydroxy metabolites was not determined. The incubations were terminated by addition of 0.8 vol of glacial acetic acid, which would readily decompose any bisallylic hydroxy fatty acids present. It therefore seems promising to investigate the CYP1A, CYP2C, and the CYP3A subfamilies for bisallylic hydroxylases.

Whether bisallylic metabolites of arachidonic acid were formed by β-naphthoflavone-induced P450 has not been investigated. Purified hepatic P450 from β-naphthoflavone-treated rabbits and rats mainly formed 19-HETE (36–38), while more recent studies have demonstrated that C_{16} to C_{18} of arachidonic acid can be hydroxylated by microsomes of β-naphthoflavone, 3,4,5,3′,4′-pentachlorobiphenyl- or 3-methylcholanthrene-treated rats (39,40).

The tissue and species distribution of bisallylic P450-hydroxylases has not yet been studied systematically. Hepatic microsomes of several species contain bisallylic hydroxylase activity (27). It remains to be determined whether these enzymes are present in kidney cortex, the lung, or in other organs rich in P450.

Allylic and bisallylic hydroxy fatty acids are formed by lower organisms. 11*R*-HODE, 13-HETE, and 13-HEPE have been found in red algae, *L. corallioides et calcareum* (25,41), while 8*R*-HODE and its precursor hydroperoxy precursor, 8*R*-HPODE, are formed by two fungi, *Gaeumannomyces graminis* and *Laetisaria arvalis* (22,42–44). The fungal biosynthesis of 8*R*-HPODE is catalyzed by linoleic acid 8*R*-dioxygenase, which is a hemoprotein (45). The algae biosynthesize the bisallylic hydroxy fatty acids by incorporation of oxygen derived from water and thus by enzymes other than mono- or dioxygenases (25,46).

METABOLIC FATE

Fatty acid epoxides and their vicinal diol products are rapidly incorporated into phospholipids. Whether bisallylic hydroxy fatty acids can be incorporated into phospholipids has not been determined. It is unknown whether metabolites formed by bisallylic hydroxylations (11-HODE, 13-HETE, 10-HETE, 7-HETE, etc.) are of quantitative importance in the breakdown of linoleic and arachidonic acids. The bisallylic hydroxy fatty acids formed by liver microsomes of phenobarbital-treated rats are mainly racemic (21,28), which argues against a role as autacoids. More knowledge in this area is likely to follow in the near future.

EPOXIDATION

Epoxides of olefins were demonstrated in 1970 to be obligatory intermediates in the metabolism of olefins to 1,2-diols by rat liver microsomes (47). Epoxidation of oleic acid to *cis*-9,10-epoxystearic acid by liver microsomes, NADPH and molecular oxygen, with subsequent enzymatic hydrolysis to *threo*-9,10-dihydroxystearic acid, was reported by Watabe *et al.* (48,49).

The first structural analysis of mammalian P450 metabolites of arachidonic acid was performed in the rabbit kidney (50). Microsomes of rabbit renal cortex and NADPH were found to metabolize [^{14}C]arachidonic acid extensively to a large series of metabolites. Two of the major metabolites were identified as 14,15-dihydroxy-5,8,11-eicosatrienoic (14,15-DiHETrE) and 11,12-DiHETrE. Two epoxides, 14,15-EpETrE and 11,12-EpETrE, were postulated as intermediates in their biosynthesis. In addition, metabolites of arachidonic acid formed by ω1- and ω2-hydroxylation and by ω-oxidation were also identified.

The biosynthesis of the two diols by renal cortical microsomes required NADPH and it was not supported by NADH (50). Carbon monoxide inhibited the biosynthesis. Liver microsomes and NADPH catalyzed the same reaction, and the mechanism of biosynthesis of 14,15-DiHETrE and 11,12-DiHETrE were investigated in rabbit liver microsomes (51). Biosynthesis under an atmosphere of $^{18}O_2$ led to incorporation of only one ^{18}O atom into the diols. This suggested that the diols were formed from epoxides by hydrolysis, according to the established mechanism of diol formation (47–49). In addition to the diols, trihydroxy metabolites were also identified as polar metabolites in both rabbit renal cortex and in liver microsomes (51,52). These products were formed by ω1- and ω2-hydroxylation of 14,15-DiHETrE and 11,12-DiHETrE.

Three issues remained to be solved regarding the microsomal biosynthesis of diols. First, the postulated epoxide intermediates should be isolated in order to prove the oxygenation mechanism. Second, it should be determined whether these metabolites were formed by intact cells, whole organs, and *in vivo*. Third, the P450 gene families of constitutive P450-epoxygenases of arachidonic acid should be determined.

The first question was solved with isolation of the four epoxides of arachidonic acid. They were isolated either from microsomal preparations with little epoxide hydrolase activity, with the aid of epoxide hydrolase inhibitors, or from purified phenobarbital-induced rat and rabbit liver P450 (CYP2B1 and CYP2B4, respectively) in a reconstituted system (36). Two novel diols, 5,6-dihydroxy-8,11,14-eicosatrienoic acid (5,6-DiHETrE) and 8,9-dihydroxy-5,11,14-eicosatrienoic acid (8,9-DiHETrE), were also identified. Epoxides of arachidonic acid were converted by purified microsomal epoxide hydrolase to diols. Rabbit liver and renal cortical microsomes also formed small amounts of 5,6- and 8,9-DiHETrE (23). The isolation of epoxides formed from arachidonic acid by rat liver microsomes was also reported (53). Falck *et al.* (54) then determined the absolute configuration of epoxides formed by phenobarbital-induced, purified rat liver P450. With the exception of 5,6-epoxy-8,11,14-eicosatrienoic acid (5,6-EpETrE), the products were formed with significant stereoselectivity. For example, 14,15-EpETrE

consisted of 80% *R,S* and 20% *S,R*, while 8,9-epoxy-5,11,14-eicosatrienoic acid (8,9-EpETrE) and 11,12-EpETrE were almost enantiomerically pure (97% of the *R,S* and *S,R* enantiomers, respectively). These results were important, since racemic epoxides of fatty acids can be formed by nonenzymatic peroxidation (55).

Rat hepatocytes and renal cells were found to metabolize arachidonic acid to 14,15-DiHETrE and 11,12-DiHETrE (23). These diols were detected in rabbit liver and rabbit renal cortex and appeared to be formed endogenously. Epoxides of arachidonic acid were also detected in rabbit kidney (56), human urine (57), human plasma (58), rat liver (59), rat urine (60), and rat plasma (61). DiHETrE were also excreted in human urine (61). However, epoxides can also be formed nonenzymatically, and all these studies did not conclusively demonstrate enzymatic biosynthesis of epoxides *in vivo*.

Evidence for *in vivo* formation of epoxides of arachidonic acid in the liver and renal cortex was soon provided. Karara *et al.* (62) determined the absolute configuration of endogenous epoxides of the liver of untreated or phenobarbital-treated rats by chiral HPLC. The epoxides were derived from hepatic phospholipids by mild alkaline hydrolysis. 8,9-EpETrE and 11,12-EpETrE consisted mainly of the *S,R* enantiomers (79 and 63% of untreated and 99 and 83% in phenobarbital-treated livers, respectively) and 14,15-EpETrE of the *R,S* enantiomer (77 and 94%, respectively). Phenobarbital treatment thus had a large effect on the stereochemistry of the epoxides of hepatic phospholipids. This indicated *in vivo* biosynthesis. Except for the enantiomeric composition of 8,9-EpETrE, there was good agreement between the enantiomers in phospholipids and the enantiomers formed by phenobarbital-induced rat liver microsomes (54). Liver microsomes of untreated and phenobarbital-treated rats formed different enantiomers of 11,12-EpETrE *in vitro*. 11*R*,12*S*-EpETrE was the major enantiomer (81%) in control microsomes and 11*S*,12*R*-EpETrE the major product (83%) by phenobarbital-induced microsomes (33).

In the human kidney cortex (63), 8,9-EpETrE was racemic and the *R,S* enantiomers of 11,12-EpETrE and 14,15-EpETrE dominated (80 and 66%, respectively). 14,15-EpETrE was the major epoxide (44%), followed by 11,12-EpETrE (33%) and 8,9-EpETrE (23%). Karara *et al.* (64) also demonstrated an enantioselective preference for incorporation of 8*S*,9*R*-EpETrE, 11*S*,12*R*-EpETrE, and 14*R*,15*S*-EpETrE at *sn*-2 of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol of rat liver. These enantiomers were also identified after hydrolysis of phospholipids.

The third question was to identify the constitutive P450 gene families which contain arachidonic acid epoxygenases. This is still under investigation, and interesting results have emerged in the last years. The first approach was to examine purified P450 for epoxygenase activity in comparison with microsomes. The studies above showed that renal and hepatic microsomes of rats and rabbits predominantly oxygenated the 14,15 and 11,12 double bonds of arachidonic acid, while the 5,6 and the 8,9 double bonds were less prone to be oxygenated (50–52,54). Monkey liver microsomes yielded this pattern as well (65). In contrast, phenobarbital-induced liver microsomes and purified phenobarbital-induced P450 of rats (CYP2B1), rabbits (CYP2B4), and pigs oxygenated all four double bonds equally (36,53). Purified P450 from human livers of deceased with unknown drug histories were also found to epoxidize all double bonds of arachidonic acid (66). Only recently have P450 which differ from these patterns of oxygenation been found.

An arachidonic acid epoxygenase was purified from rabbit renal cortex and designated P450 2CAA (67). P450 2CAA formed 11,12-EpETrE (about 45% of total metabolites) and 14,15-EpETrE (about 30% of total metabolites) as the two major products. These results are interesting since they demonstrate some regiospecific oxygenation of the 11,12 double bond of arachidonic acid. Laethem and Koop (68) showed that rabbit CYP2C1, transiently expressed in COS-1 cells, catalyzed biosynthesis of 11,12-EpETrE, 14,15-EpETrE and 19-HETE (about 48, 16, and 13%, respectively), while rabbit CYP2C2 in the same system yielded only the two epoxides (11,12-EpETrE and 14,15-EpETrE) in a ratio of 2:1, respectively. P450 2CAA and CYP2C2 formed the epoxides with some stereoselectivity, 63 and 61% 11*S*,12*R*-EpETrE, respectively, and 72 and 62% 14*R*,15*S*-EpETrE, respectively (69). Purified P450 2CAA had a slightly larger molecular weight than CYP2C2 on polyacrylamide gel electrophoresis, but identical amino-terminal sequence. It seems likely that P450 2CAA and CYP2C2 are very similar but not identical isoforms (68).

The epoxygenases of the rat and human kidney cortex have been investigated (60,70). Rat kidney microsomal epoxygenase formed 8*R*,9*S*-, 11*R*,12*S*-, and 14*S*,15*R*-EpETrE with optical purities of 97, 88, and 70%, respectively. Inhibition studies with antibodies against P450 isoforms indicated that the epoxygenase belonged to the CYP2C gene family. The renal epoxygenase was cloned and found to be virtually identical with rat liver CYP2C23 (71). Expressed in COS-1 cells, the recombinant rat kidney epoxygenase formed the three epoxides with virtually identical optical purity with that of renal cortical microsomes (95, 85, and 75%, respectively). 11,12-EpETrE was the main product (54%), followed by 8,9- and 14,15-EpETrE (27% and 14%, respectively). In the human kidney cortex, CYP2C8 was identified as one of the P450-epoxygenase isoforms. Metabolism of arachidonic acid by recombinant CYP2C8 was both regio- and enantioselective for 11*R*,12*S*- and 14*R*,15*S*-EpETrE.

The identification of the CYP2C gene family as constitutive renal epoxygenases, which oxygenate the 11,12- and 14,15-double bonds with enantioselectivity, is of physiological interest. Rat renal cortical epoxygenase activity is induced by salt loading (60). Whether salt loading will induce epoxygenases in kidneys of other species is unknown, but this interesting finding in the rat suggests additional studies are necessary.

Site-directed mutants of CYP2B1 have been investigated

as epoxygenases of arachidonic acid (72). The substrate specificity of a P450 can be altered by an amino acid mutation at a few key positions in the substrate-heme pocket (73,74). CYP2B1 predominantly produced 14,15-EpETrE, but mutation of two important amino acids, Ile-114 or Gly-478, had profound effects on the biosynthesis of epoxides. For example, double mutation of CYP2B1 with Ala at these two positions yielded an active enzyme, which formed 8,9- and 5,6-EpETrE as the two main products. These elegant results suggest that Ile-114 and Gly-478 may change the orientation and oxygenation of the fatty acid in the active site. In regard to the mechanism of formation of epoxides, Groves *et al.* (75) have shown that epoxidation of propylene by CYP2B4 occurs with a concomitant, stereospecific hydrogen/deuterium exchange from the aqueous milieu. Epoxides of fatty acids could be expected to be formed in the same way, but this has not been determined.

An epoxygenase of arachidonic acid has also been purified from chick embryo livers after pretreatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or β-naphthoflavone, both of which potently induce the epoxygenase activity (76–78). This P450 was immunologically different from the phenobarbital-induced arachidonic acid epoxygenase, but the catalytic properties were not investigated in detail. It should be noted that β-naphthoflavone induces ω2-hydroxylation of fatty acids (see above) in other species (13), while the chick epoxygenase apparently is induced by Ah receptor dependent inducers of P450 (76–78). Whether epoxides of arachidonic acid also mediate or modulate manifestation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxicity, which has been suggested (76), remains to be established.

Liver microsomes of rats and rabbits can epoxidize a large number of different C_{18}–C_{22} unsaturated fatty acids. Epoxidation of oleic acid to *cis*-9,10-epoxystearic acid was described in 1971 (48). Linoleic acid, α-linolenic acid, 5,8,11-eicosatrienoic acid, 5,8,11,14,17-eicosapentaenoic acid, and docosahexenoic acid can be oxygenated to epoxides/diols (21,24,79,80). The double bond closest to the ω-end of many substrates has been readily oxygenated by liver microsomes of untreated animals.

Epoxidation of oleic, linoleic, α-linolenic, and γ-linolenic acids by CYP2C2 and CYP2CAA were reported recently (81). Linoleic and α-linolenic acids were converted to an equal mixture of all possible epoxides, while γ-linolenic acid was oxygenated at the 9–10 and 12–13 double bonds but not at the 6–7 double bond. Data on the epoxygenation of other unsaturated fatty acids by members of the CYP2C gene family is likely to follow. So far, the only strictly position-specific epoxygenase has been found in monkey seminal vesicles. In the presence of NADPH, microsomes of this tissue converted eicosapentaenoic acid into 17*S*,18*R*-epoxy-5,8,11,14-eicosatetraenoic acid (17*S*,18*R*-EpETE) and the corresponding diol, 17,18-dihydroxyeicosatetraenoic acid (65). Arachidonic acid was converted to 18*R*-HETE. It is possible that this seminal vesicle P450 could be identical with prostaglandin E 19*R*-hydroxylase (82).

P450-EPOXYGENASES AND BIOLOGICAL EFFECTS

EpETrE can affect cellular function in a wide variety of different tissues and cells. The mechanism behind these biological effects appears to be largely unknown. There appears to be little evidence for existence of specific receptors for EpETrE on cell membranes. In a few cases, the effects of EpETrE can be explained by inhibition of cyclooxygenase, by metabolism by cyclooxygenase, or by effects on other enzymes of the arachidonic acid cascade. When high micromolar concentrations of EpETrE are used, nonspecific membrane actions could contribute. Some investigators report effects at nanomolar concentrations, which might be of physiological relevance and deserve further attention. The hyperpolarizing action of EpETrE in certain cells is of considerable interest.

Liver. Not much is known about the biological effects of EpETrE on liver function. Most reports examine effects of micromolar concentrations. 14,15-EpETrE (10 to 50 μM) has been postulated to be involved in vasopressin-stimulated glycogenolysis in rat liver hepatocytes *via* an increase in phosphorylase a activity stimulated by cytosolic calcium increase (83). 14,15-EpETrE (1–10 μM) also stimulated ADP-ribosylation of a 52 kDa protein in rat liver cytosol, and its metabolite 14,15-DiHETrE was almost inactive (84). Whether these effects are stereospecific for one of the two enantiomers is unknown. Further information on biological roles of EpETrE on liver function may be obtained if studies are carried out in the presence and absence of clotrimazole and ketoconazole, which have been shown to be potent inhibitors of rat hepatic epoxygenase activity *in vitro* (85). Another unexplored area is the effects of P450 metabolites of arachidonic acid on the peroxisome proliferator-activated receptor (PPAR), the nuclear hormone receptor that regulate gene transcription in response to peroxisome proliferators. PPAR is activated by many eicosanoids, e.g., by PGA, PGD and PGJ compounds, and 8*S*-HETE (86).

Kidney. This research area has been summarized in excellent reviews by McGiff and collaborators (87,88), who emphasized that P450 metabolites of arachidonic acid are formed in critical nephron segments and may participate in receptor-mediated signal transduction as second messengers.

In many species, renal cortical epoxygenase activity is high and has been localized to specific cells of the nephron (89,90). Major products are 11,12- and 14,15-EpETrE in the rabbit (68) and 8,9-, 11,12-, and 14,15 EpETrE in the rat (60). As renal epoxygenase activity is of the same order of magnitude as in liver, the biological importance of EpETrE in the kidney needs to be determined. While suggested roles include modulation of the effects of renal hormones, regulation of blood flow, and of tubular function, a precise role in kidney physiology has not yet been defined (87). Roles of these metabolites have nevertheless been proposed in several models including experimental hypertension in the rat. However, there were no statistically significant differences in epoxygenase activity between normal and hypertensive rats at any age (91). In women with pregnancy-induced hyperten-

sion, increases in urinary 11,12- and 14,15-DiHETrE were observed (61).

Both racemic EpETrE and their dihydroxy metabolites inhibited the vasopressin-stimulated osmotic water flow in toad bladder *via* inhibition of adenylate cyclase, with 5,6- and 11,12-EpETrE being most potent. However, 10 μM concentrations were required for activity (92). Interestingly, 14,15-DiHETrE (0.1 μM) was as potent as PGE_2 in inhibiting the action of vasopressin on collecting ducts of the rabbit (93). 5,6-EpETrE (10 nM–1 μM) caused a transient increase in intracellular calcium of proximal tubular epithelium, mimicking the actions of angiotensin II (AII) and suggesting that P450 metabolites are involved in AII-induced calcium mobilization (94). In addition, racemic 14,15-EpETrE (1 μM) has been found to inhibit renin release from cortical slices of rat kidney (95). As 14*S*,15*R*-EpETrE is the major enantiomer of 14,15-EpETrE formed by renal microsomes and by the renal epoxygenases of rabbits and rats (60,68), it will be of interest to determine whether the effects on vasopressin and the renin-AII system are stereospecific.

Infusion of arachidonic acid into the rat renal artery causes vasoconstriction, which is blocked by inhibitors of PGH synthases, revealing a vasodilator response (96). This vasodilatation appeared to be dependent on endothelial cytochrome P450 as judged from the effects of inhibitors of P450 (96,97). In the isolated rabbit kidney, 5,6-EpETrE, 8,9-EpETrE, and 11,12-EpETrE induced vasodilatation, while 14,15-EpETrE induced vasoconstriction (98). The 5,6-epoxide was most potent and partly dependent on PGH synthases for vasoactivity (99,100). A recent detailed analysis showed that the effect of the 5,6-epoxide on the renal vasculature differed in the buffer and the blood perfused rat kidney model (101). In the latter, 5,6-EpETrE exerted vasoconstrictor effects, presumably due to metabolism by PGH synthase and thromboxane synthase of platelets to a vasoconstrictor substance. This action was antagonized by indomethacin. In the isolated rat kidney perfused with physiological buffer, the epoxide was a vasodilator and indomethacin was without effect on this response.

These observations thus suggested that epoxides may modulate renal function. The renal P450 system should be influenced by variations in salt and water balance or by other physiological means. Epidermal growth factor (30 nM) increased endogenous EpETrE levels fivefold within 10 min in primary cultures of rabbit proximal tubule cells and one epoxide, 5,6-EpETrE, augmented the effect of EGF on thymidine incorporation (102). The only physiological stimuli, which has been shown to increase renal epoxygenases, is salt loading, which results in a ten- to twelvefold increase in urinary excretion of arachidonic acid metabolites (EpETrE and DiHETrE) in rat urine (69). CYP2C23 appears to be the predominant, constitutive epoxygenase in the rat kidney while salt loading induces a yet unidentified CYP2C isoform that seems to be different from CYP2C23 (60,68,71,103).

Lung. There are species differences with respect to the production of epoxygenase metabolites by lung tissue. An early report indicated that rabbit lung tissue contained relatively little epoxygenase activity, forming only small amounts of 11,12-DiHETrE (23), in comparison with rabbit liver and kidneys. However, epoxygenase metabolites have been found in the rabbit and guinea pig lung, and CYP2B4 has been identified as a lung epoxygenase (104,105). The role of these metabolites in the lung is unclear. Methylated 5,6-EpETrE and 8,9-EpETrE relaxed guinea pig hilar bronchi *in vitro,* and epoxides could also be demonstrated in bronchoalveolar lavage fluid (105). Leukocytes in lung lavage from rats exposed to hyperoxia formed epoxides of linoleic acid, 12,13-epoxyoctadecenoic and 9,10-epoxyoctadecenoic acids, and these metabolites have been designated leukotoxin (106). Leukotoxin uncouples mitochondrial respiration and relaxes rat stomach smooth muscle at high concentrations (20–160 μM) (106). These products have also been identified in humans, but their biological significance is unknown (107). Induction of the cytochrome P450 system with β-naphthoflavone did not alter guinea pig pulmonary venule contractions induced by hypoxia or anoxia (108).

Platelets. The first report indicating a biological function of P450 metabolites of arachidonic acid appeared in 1976. Cinti and Feinstein (109) found that two inhibitors of P450 (metyrapone and CO) inhibited arachidonic acid induced platelet aggregation and the formation of aggregation factors from arachidonic acid by platelet microsomal P450. In spite of this report, it seems most unlikely that platelets can form EpETrE enzymatically. Convincing biosynthesis of EpETrE by platelets has not been demonstrated, but EpETrE have been identified in phospholipids of human platelets where they can be released by thrombin or platelet-activating factor (110). These EpETrE are either formed nonenzymatically or by other cells and taken up by platelets. EpETrE can affect platelet function. All EpETrE inhibited arachidonic acid-induced aggregation of washed platelets at 1–10 μM (see Ref. 17 for review). The antiaggregatory effects of EpETrE were recently attributed to selective inhibition of Ca^{2+} entry into platelets, as *cis*-14,15-EpETrE reduced both receptor-independent discharge by thapsigargin or receptor-dependent discharge by thrombin; however, these effects utilized high concentrations of *cis*-14,15-EpETrE (60 μM) (111). Interestingly, an analogue of 14,15-EpETrE, *cis*-4,15-episulfideeicosatrienoic acid, preferentially inhibited ionophore A23387-induced platelet aggregation, but did not inhibit aggregation induced by thrombin (112). 14,15-EpETrE (0.3 mg/kg i.v.) also inhibited platelet aggregation in mouse cerebral arterioles (113).

Platelets can also metabolize 5,6-EpETrE by PGH synthase-1 and thromboxane synthase. Balazy (112) demonstrated that human platelets metabolized 5,6-EpETrE into a number of metabolites, e.g. 5-hydroxy-6,9-epoxy-TXB_1, 5,6-epoxy-12-hydroxyheptadecadienoic acid, and 5,6-epoxy-12-hydroxyeicosatrienoic acid. It seemed likely that 5-hydroxy-6,9-epoxy-TXB_1 was formed from 5,6-epoxy-TXB_1 (and 5,6-epoxy-TXA_1) by intramolecular rearrangement in analogy with the formation of two isomers of 5-hydroxy-PGI_1 from 5,6-epoxy-$PGF_{1\alpha}$. Platelet aggregation was not induced by

5,6-EpETrE, although following preincubation, responsiveness to arachidonic acid was reduced. The author suggests that the platelet metabolites may be useful in assessing production of 5,6-EpETrE in humans (114).

The effects of other epoxides on platelet aggregation and thromboxane synthesis were recently investigated by VanRollins (115). In contrast to the arachidonic acid epoxides, all of the regioisomers of eicosapentaenoic and docosahexaenoic acid epoxides were found to inhibit platelet aggregation at much lower concentrations than those needed for blocking thromboxane biosynthesis. When eicosapentaenoic and docosahexaenoic acids are given as dietary supplements, platelet aggregation is impaired but thromboxane synthesis is unaffected. The author suggests that multiple circulating epoxides of these two fatty acids may contribute to platelet inhibition after eating fish oils.

Vascular tree. The effects of arachidonic acid and its P450 metabolites on the vasculature should be considered in the context of factors which mediate endothelium-dependent relaxation. In addition to prostacyclin and nitric oxide, which is the best characterized endothelium-derived relaxing factor, there may be at least one additional vasodilator substance, which has not yet been identified. This putative substance is designated endothelium-derived hyperpolarizing factor (EDHF), and it may be important for vasoregulation particularly of small resistance arteries (Refs. 116–118 for review). EDHF may be less reactive than nitric oxide and distinct from prostanoids. An increase in free calcium may release EDHF, which may increase conductance to potassium in smooth muscle cells. Some investigators suggest that EpETrE might be candidates of EDHF (119), while others remain skeptical (120).

Exogenous EpETrE and their vicinal diol products are rapidly taken up by arterial smooth muscle cells and by endothelial cells *in vitro* (121–123). The epoxides are partly hydrolyzed to vicinal diols and released to the medium. The metabolism of 11,12-EpETrE by arterial smooth muscle cells was recently studied by Spector and collaborators (124). The vicinal diol, 11,12-DiHETrE, and a novel metabolite, 7,8-dihydroxyhexadecadienoic acid were identified. The latter was apparently formed from 11,12-DiHETrE by two steps of β-oxidation. Interestingly, both metabolites (1–5 μM) caused vasorelaxation of precontracted porcine coronary artery rings.

Several investigators have found that arachidonic acid induced endothelium-dependent relaxation of the rabbit aorta (123), pulmonary rings (125), and coronary arteries (127). SKF 525A (a P450 inhibitor) blocked this effect (125–127), but it should be remembered that this inhibitor may have direct effect on potassium conductance (116).

5,6-EpETrE reduced vascular resistance of the isolated perfused rat caudal artery, the other epoxides being inactive (128). The biological activity of 5,6-EpETrE was dependent on PGH synthase in rat caudal artery and cerebral arterioles (128,129). It seems likely that the effects of 5,6-EpETrE were partly due to metabolism to epoxyprostaglandins. Carroll *et al.* (99) found that 5,6-EpETrE was converted to 5,6-epoxy-PGE_1 after circulating through the rabbit kidney. Authentic 5,6-epoxy-PGE_1 was equipotent to PGE_2 as a vasodilator in the isolated, perfused rabbit kidney. Metabolism by cyclooxygenase has also been implicated in the vasodilatory effects of 8,9-EpETrE and 11,12-EpETrE on intestinal serosal blood flow (130). 5,6-EpETrE may also be an important mediator of calcium influx in human and bovine endothelial cells (131). The vascular effects of 5,6-EpETrE and the other P450 metabolites of arachidonic acid have been reviewed (87,132). Systemic administration of EpETrE has virtually no effect on systemic or cerebral blood flow in the rat (133).

Epoxides of arachidonic acid may also affect vascular tone by PGH synthase-independent mechanisms. 8,9-EpETrE and 11,12-EpETrE caused transient dilatation of precontracted cerebral arteries at 50 μM and also increased the frequency of opening of K^+ channels at 5 μM (134). All four EpETrE (0.1–3 μM) increased the open-state probability of the Ca^{++}-activated K^+ channel (K_{Ca} channel) in cells from rabbit portal vein, rat caudal artery, guinea pig aorta, and porcine coronary artery (119,135). 11,12-EpEtTrE hyperpolarized and increased the open-state probability of a K_{Ca} channel in coronary smooth muscle cells (119). In renal vascular muscle cells 11*R*,12*S*-EpETrE (1–100 nM) increased the activity of the K_{Ca} channel (136). This enantiomer also relaxed small renal arteries preconstricted with phenylephrine. The vasodilator effect of some EpETrE may therefore involve activation of a K_{Ca} channel.

Vessels with injured endothelium due to artery stenosis synthesize 5,6-, 8,9-, 11,12-, and 14,15-EpETrE (137). The authors suggest that these metabolites may counteract the vasoconstriction action of thromboxane A_2 (137). 14,15-EpETrE ($2.5–5 \times 10^{-7}$ M) promotes adhesion of U937 cells to endothelium, suggesting that this compound may play a role in inflammation or atherogenesis (138). Rabbit aorta, isolated from animals with experimental hypercholesterolemia, was also found to generate all four EpETrE, but normal rabbit aorta failed to do so. All four regioisomers at concentrations from 10^{-8} to 10^{-5} M relaxed norepinephrine-precontracted aorta from normal and cholesterol-fed animals. However, only 14,15-EpETrE decreased the basal, but not the stimulated synthesis of 6-keto $PGF_{1\alpha}$ by aortas from normal rabbits (139).

Cultured bovine coronary artery endothelial cells form cyclooxygenase, lipoxygenase, and P450 metabolites of arachidonic acid (140). All four EpETrE were identified, and they may contribute to the endothelium-dependent relaxation caused by bradykinin and arachidonic acid. However, a recent study found that arachidonic acid metabolites were not involved in acetylcholine-induced endothelium-dependent hyperpolarization in guinea pig corotid artery (120). Further studies are needed to clarify the physiological role of P450 and EpETrE in the vascular wall.

Heart. The vasodilation of bradykinin on coronary arteries in the rat heart also seemed to be dependent on P450 metabolism of arachidonic acid (141). Effects of EpETrE on isolated hearts and ventricular myocytes have been reported (142).

These compounds did not affect cardiac or coronary functions in normal isolated guinea pig heart, but 11,12-EpETrE depressed recovery of the contractility after ischemia and elevated Ca_i of ventricular myocytes. The functional significance of the elevation of Ca_i remains to be determined. P4501A1 has been found in fish heart tissue (143). Recently, a novel arachidonic acid epoxygenase, designated P4502J2, was found in the human heart (144). This enzyme forms the 5,6-, 8,9-, 11,12-, and 14,15-EpETrE with little regioselectivity in the following amounts: 21, 24, 18, and 37%, respectively. The 8,9-, 11,12-, and 14,15-EpETrE could also be demonstrated in heart tissue (142).

Central nervous system and pituitary. P450 is present in many parts of the central nervous system (3,145), but only recently has biosynthesis of arachidonic acid epoxides been reported (146). Slices of mouse brain formed two epoxides, 14,15-EpETrE and 5,6-EpETrE, but their enantiomeric composition was not determined. Brain-synthesized 5,6-EpETrE caused transient cerebral arteriolar dilation similar to that caused by synthetic 5,6-EpETrE (15.6 μM), and the authors suggest it may have a role in cerebral microcirculation regulation (146). Microsomes of cat brain formed large amounts of 5,6-, 8,9-, and 11,12-EpETrE and small amounts of 14,15-EpETrE. 8,9-EpETrE and 11,12-EpETrE (50 μM) dilated serotonin-preconstricted, pressurized cerebral arteries, presumably through activation of K^+ channels (134). Rabbit cerebral arteries were dilated by 5,6-EpETrE (15.6 μM) while 8,9-EpETrE, 11,12-EpETrE, and 14,15-EpETrE were less active. The suggested mechanism was *via* cyclooxygenase metabolism of 5,6-EpETrE with formation of dilator oxygen radicals (129).

A series of recent papers have demonstrated that rat brain astrocytes in primary culture produce EpETrE (146–148). 11,12-EpETrE and 14,15-EpETrE were identified in one study as well as mRNA of P450 2C11 in the astrocytes (148). Ellis and collaborators (149) also report synthesis of 5,6-EpETrE and 14,15-EpETrE and the corresponding vicinal diols. These studies further emphasize that arachidonate epoxygenses are present in the brain, and that it is conceivable that EpETrE could modulate K_{Ca} channel activity in the cerebral microvessels.

Microsomes of rat hypothalamus oxygenated arachidonic acid to epoxides in the presence of NADPH (150). In addition, 5,6-EpETrE (2.5 μM) stimulated the release of somatostatin from the median eminence *in vitro*. These results were extended by demonstration and quantification of endogenous 8,9-, 11,12-, and 14,15-EpETrE in the rat hypothalamus. In contrast to 5,6-EpETrE, 8,9-EpETrE released somatostatin from the median eminence with an EC_{50} of only 5 pM (151). This profound bioactivity is remarkable, and it should therefore inspire further investigations.

P450 metabolism of arachidonic acid also occurs in the pituitary. Microsomes of rat anterior pituitary formed 20-HETE and at least three epoxides of arachidonic acid (5,6-, 11,12-, and 14,15-EpETrE) (152). These P450 metabolites affect anterior pituitary cells at relatively high concentrations. Inhibitors of P450 (SKF525A, piperonyl butoxide) blocked the release of corticotropins in response to a variety of secretagogues in mouse pituitary tumor cells (153). 5,6-EpETrE (1 μM) caused efflux of calcium and release of luteinizing hormone (154), stimulated release of prolactin from a rat anterior pituitary cell line, GH3 cells, at a concentration of 50 μM (155), and stimulated the release of growth hormone from pituitary cells in culture at a concentration of 20 μM (156). In addition, 5,6-, 8,9-, 11,12-, and 14,15-EpETrE (3 μM) all stimulated release of growth hormone from a population of enriched somatotrophs, with 5,6-EpETrE and 14,15-EpETrE more potent than the other two regioisomers (157).

Microsomes of the neural lobe of rat pituitary metabolized arachidonic acid to the major metabolites 8,9-, 11,12-, and 14,15-EpETrE which were produced in equal quantities and stimulated release of oxytocin and vasopressin over a concentration range from 10^{-8} to 10^{-6} M (158). These concentrations were 1.5 times greater than that of PGE_2 for similar effects on oxytocin and vasopressin release. 5,6-EpETrE was produced in lower quantities and was biologically inactive.

These studies demonstrated that epoxides of arachidonic acid were formed by mouse and cat brain, and by rat hypothalamus, pituitary, and astrocytes. Little is known about the stereochemistry of the products and the P450 isozymes catalyzing their biosynthesis. Because of the high concentrations used in some of these studies, the physiological function of arachidonic acid epoxides in regulation of hormone release from hypothalamus or the pituitary still remains to be established (see Ref. 159 for review).

Adrenals, pancreas, and ovaries. There are few reports on P450 metabolism of arachidonic acid by endocrine glands. EpETrE, HETE, and prostaglandins were identified as metabolites of arachidonic acid formed by rat adrenal glomerulosa cells (160), where they presumably have a role in aldosterone release (161). Epoxyeicosatrienoic acids have been shown to release insulin and glucagon from pancreatic islets (162). Human granulosa-luteal cells in culture and a human chorioncarcinoma cell line (BeWo) formed 14,15-EpETrE and 14,15-DiHETrE (163). Human granulosa cells of the ovary metabolized arachidonic acid to epoxides, and 14,15-EpETrE stimulated estradiol production at low concentrations (1–50 nM); however higher concentrations (10–50 μM) inhibited estradiol production and hCG-stimulated estradiol secretion. 14,15-EpETrE did not affect progesterone production at any concentration tested (164). 14,15-EpETrE is produced by human amnion, trophoblast, and umbilical vein tissues and may be involved in maintenance of vascular function (165).

Conclusion. A wide range of biological effects of epoxygenase metabolites of arachidonic acid and a few other fatty acids have been described in liver, kidney, lung, platelets, cardiovascular, nervous, and endocrine systems. Some studies have used micromolar concentrations of P450 metabolites, while other studies report effects in the nanomolar concentration range as P450 metabolites of arachidonic acid are present in low cellular concentrations, it remains to be determined

whether these metabolites are formed in high enough concentrations to be of physiological importance.

METABOLISM OF EPOXY AND BISALLYLIC HYDROXY FATTY ACIDS

Epoxide hydrolases. Epoxides of arachidonic acid were converted by epoxide hydrolases of hepatic microsomes to vicinal diols (36). Experiment with biosynthesis of epoxides using hepatic microsomes under $^{18}O_2$ gas showed that one ^{18}O atom was present in the diols. Gas chromatography–mass spectrometry (GC–MS) analysis showed that both ^{16}O and ^{18}O were found at the hydroxyl at C_{14} or at C_{15} of 14,15-DiHETrE and at the hydroxyl at C_{11} or at C_{12} of 11,12-DiHETrE (51). This indicated that oxygen from water or enzyme could be added to both carbons of the oxirane rings during the enzymatic hydrolysis.

Purified rat liver microsomal epoxide hydrolases were found to hydrolyze the four epoxides of arachidonic acid with V_{max} between 0.09 and 0.25 μmol/min/mg protein (36), but K_m values were rather high, e.g., 0.16 mM for 14,15-EpETrE. Corresponding figures for hydrolysis of these epoxides by purified mouse liver cytosolic epoxide hydrolase were between 0.07 and 1.26 μmol/min/mg protein, but the K_m value for 14,15-EpETrE was only 8 μM (166). Both microsomal and cytosolic epoxide hydrolases can thus hydrate the epoxides, but the cytosolic enzyme appeared to be more efficient (higher V_{max} and lower K_m). The fact that some investigators report biosynthesis of epoxides from [^{14}C]arachidonic acid by hepatic microsomes, while others mainly find the corresponding diols may be due to contamination with cytosolic epoxide hydrolases and to differences in the specific activity of the radiolabeled substrate.

The hydration of *cis*-epoxyeicosatrienoic acids by cytosolic epoxide hydrolases were investigated by Zeldin *et al.* (167), using chiral HPLC to separate the *S,S* and *R,R* enantiomers of the vicinal DiHETrE. Interestingly, acid-catalyzed hydration of the *cis*-epoxides did not always lead to equal formation of the *S,S* and *R,R* isomers; 14*S*,15*R*-EpETrE yielded a 3:1 ratio of these antipodes. Soluble epoxide hydrolase of rabbit liver hydrolyzed racemic 14,15-EpETrE to a 4:1 mixture of *R,R* and *S,S* enantiomers of 14,15-DiHETrE. Incubation with 14*S*,15*R*-EpETrE, the major enantiomer formed in the rat and rabbit kidney (60,168), yielded the *S,S* and *R,R* antipodes in a 3:1 ratio. 14*R*,15*S*-EpETrE, the major enantiomer of the liver and human kidney (62,63), was hydrated mainly to the *R,R* antipode of 14,15- DiHETrE (ratio 6:1). These results show that the oxirane ring was opened by cytosolic rabbit liver epoxide hydrolase mainly by addition of oxygen at C_{15}. Similarly, epoxide hydrolase selectively catalyzed addition of oxygen at C_9 of 8,9-EpETrE, while 11,12-EpETrE was hydrolyzed without regioselectivity. From these results, it seems likely that the *R,R* enantiomer of 14,15-DiHETrE is the major enantiomer in the liver and human kidney cortex, while *S,S* enantiomer is likely to predominate in the rabbit and rat kidney. The results were recently extended to studies with soluble epoxide hydrolase and a series of epoxides, their methyl esters, and hydrogenated compounds (169).

The catalytic mechanism of soluble epoxide hydrolases has been investigated in detail by Hammock and collaborators (170). *cis*-9,10-Epoxystearic acid was used as one of two model substrates. [^{18}O]Epoxystearic acid was enzymically hydrolyzed to the diol and ^{18}O was present at either C_9 (69%) or at C_{10} (31%). The other oxygen was found to be derived from the enzyme itself. When the unlabeled epoxide was incubated with excess enzyme for only one cycle in $H_2{}^{18}O$, the diol did not contain ^{18}O. Further analysis showed that the carboxylate anion of Asp-333 of the enzyme attacked the epoxide substrates and formed an α-hydroxyacyl-enzyme intermediate, which was hydrolyzed by "activated" water (postulated *via* a histidine) to the active enzyme and the diol.

PGH synthases. Soon after their discovery, EpETrE were found to affect many different organs and cells (17,82). It seemed possible that these effects could be either direct or indirect *via* modulation of prostaglandin biosynthesis. PGH synthases form prostanoids from eicosanoic fatty acids, which contain at least three double bonds, located at the 8,9, 11,12, and 14,15 positions (171–174). 5,6-EpETrE contains these double bonds.

5,6-EpETrE was found to be rapidly metabolized by PGH synthase-1 *in vitro* into epoxy prostaglandin endoperoxides, 5,6-epoxy-PGG_1 and 5,6-epoxy-PGH_1, which were isolated (175). These endoperoxides could be reduced to 5,6-epoxy-$PGF_{1\alpha}$ or could be enzymatically isomerized to 5,6-epoxy-PGE_1 (175–177). 5,6-Epoxy-$PGF_{1\alpha}$ is chemically unstable and can undergo intramolecular rearrangement with formation of 5-hydroxy-$PGI_{1\alpha}$ and 5-hydroxy-$PGI_{1\beta}$ (178,179). The latter are biologically inactive. McGiff and colleagues (99) have shown that exogenous 5,6-EpETrE is metabolized to a vasodilator, 5,6-epoxy-PGE_1, by the isolated perfused rabbit kidney.

The 8,9-, 11,12-, and 14,15-epoxides of arachidonic acid lack the necessary double bonds for prostaglandin biosynthesis. Some of them may undergo abortive PGH synthase reactions or can inhibit this enzyme. The *R,S* enantiomer of 14,15-EpETrE inhibits PGH synthase-1 of ram vesicular glands and platelets (176,180). It seems likely that some of the pharmacological effects of 14,15-EpETrE can be attributed to inhibition of PGH synthase (157). Recent studies show that 8,9-EpETrE can be oxygenated by PGH synthase to 8,9-epoxy-11*R*-hydroxyeicosatrienoic acid (181), which might contribute to the observed effects of 8,9-EpETrE on the renal circulation (182).

GSH transferases. Leukotriene A_4 (LTA_4) is an unstable allylic epoxide of arachidonic acid and an important intermediate in leukotriene biosynthesis. LTA_4 is conjugated with reduced GSH by LTC_4 synthase (183), leading to formation of LTC_4 and ultimately other peptidoleukotrienes with potent biological activities. It was therefore of interest to determine whether the homallylic epoxides formed by P450 could be conjugated with glutathione by glutathione *S*-transferases. This was found to be the case (184). 14,15-EpETrE was con-

jugated with glutathione, and the transferase isozymes 3-3 and 5-5 were most active with K_m values of about 10 μM and V_{max} values between 25 and 60 nmol conjugate/min/mg protein. The other epoxides were also substrates but were conjugated less efficiently.

Incorporation into phospholipids. Epoxides of arachidonic acid and other PUFA can be incorporated into phospholipids. 8*S*,9*R*-, 11*S*,12*R*-, and 14*R*,15*S*-EpETrE were, for example, demonstrated at the glycerol-*sn*-2 position of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol in the liver (64). Exogenous 14,15-EpETrE is rapidly incorporated into phospholipids of mastocytoma cells. The distribution of the epoxide in phospholipids was phosphatidylethanolamine>phosphatidylcholine>phosphatidylinositol (185). The rate of release of the epoxide from phosphatidylcholine and phosphatidylinositol was higher than the release of arachidonic acid from these phospholipids by phospholipase A_2 in agreement with previous reports (55, cf. Ref. 186).

EpETrE and DiHETrE were recently found to be incorporated into phospholipids of vascular endothelial cells (121,133), smooth muscle cells (122), endothelial cells (123), and astrocytes (148). These findings should be considered in the context of these metabolites as putative EDHF.

ANALYSIS OF EPOXIDES AND BISALLYLIC HYDROXY FATTY ACIDS

GC–MS and liquid chromatography–mass spectrometry (LC–MS) analyses. GC–MS has been the primary tool for structural identification of epoxides and other P450 metabolites of PUFA (see Ref. 187 for review). The mass spectra of epoxides of PUFA provide relatively little information on the position of the epoxide. Analysis of epoxides *via* 3-pyridinylmethyl esters are more informative (188). Conversion of the epoxides to diols or chlorohydrin adducts usually gives mass spectra with distinct fragmentation (189). A large and extremely useful collection of electron impact mass spectra of eicosanoids has been compiled by Pace-Asciak (190). Instruments for GC–MS analysis and interpretation of mass spectra of fatty acid derivatives and eicosanoids have been described by Murphy in an excellent monograph (191).

Methods for analysis based on deuterated or ^{18}O labeled internal standards have been used for quantification of epoxides or the corresponding diols by mass fragmentography (see Ref. 192 for details). Recently, EpETrE and their corresponding diols were identified in nanogram or picogram quantities using capillary GC–MS in election ionization as well as in the negative and positive ion-chemical ionization modes (121,193).

Yamane *et al.* (194) systematically studied epoxides of polyunsaturated eicsoanoic fatty acids and docosahexaenoic acid by LC–MS (thermospray). In addition, mass spectra of many of the corresponding diols were reported. So far LC–MS has not been used for analysis of bisallylic hydroxy fatty acids.

Steric analysis of hydroxy fatty acids. Enantiomers of *cis-trans* conjugated HETE and other *cis-trans* conjugated hydroxy fatty acids can be separated by chiral HPLC on *R*-(−)-*N*-(3,5-dinitrobenzyol)-α-phenylglycine columns as devised by Kühn *et al.* (195). After derivatization of the hydroxyl groups, the separation can be improved (196). Brash *et al.* (28) reported that bisallylic hydroxy metabolites of PUFA show increased end absorption at 220 nm in comparison with ω1 and ω2 hydroxy metabolites. This is convenient for monitoring these metabolites. Brash *et al.* (28) also reported that the enantiomers of 7-HETE, 10-HETE, and 13-HETE were at least partly resolved by chiral HPLC on Chiralcel OD (Diacel, Tokyo, Japan). Steric analysis of bisallylic hydroxy fatty acids can also be performed after partial hydrogenation of the double bonds, synthesis of (−)-menthoxycarbonyl derivatives and ozonolysis (21,25). Steric analysis of ω4-, ω3-, and ω2-hydroxy fatty acids can be performed by separation of diastereoisomeric *S*-phenylpropionic acid derivatives on GC (197). It is also possible to partly resolve the enantiomers of 18-HETE and 19-HETE on chiral HPLC (198).

Steric analysis of epoxy fatty acids. Chiral HPLC for separation of the enantiomers of all four epoxides of arachidonic acid was developed by Hammonds *et al.* (199), using Chiralcel OB and Chiralcel OD columns (Diacel). The epoxides were analyzed as methyl esters or as pentafluorobenzyl esters. The resolution was improved by hydrogenation. This valuable technique has now been used in many studies from this group. Whether these columns also can be used for steric analysis of epoxides of other fatty acids has not been determined. Cellulose derivatized with trisphenylcarbamate (Chiralcel OC, Diacel), for example, resolved the enantiomers of some epoxides of arachidonic, linoleic, and α-linolenic acids (168), but the enantiomers of the ω3 epoxides of eicosapentaenoic acid or linolenic acid were not separated. A general method for finding the suitable chiral HPLC column is lacking. There is a large selection of different chiral HPLC columns available (200), yet the choice of HPLC column is empirical, and, unfortunately, these HPLC columns with derivatized cellulose are very expensive.

A general method for steric analysis of *cis* and *trans* epoxides of fatty acids was developed by Fahlstadius and Hamberg (201). This method was based on hydrogenation and conversion of the epoxide to two regioisomeric allylic alcohols, followed by ozonolysis of (−)-menthoxycarbonyl derivatives and GC–MS analysis.

Steric analysis of vicinal diols. Vicinal *threo*-DiHETrE, formed by hydrolysis of EpETrE, can also be resolved by chiral HPLC as methyl ester derivatives using Chiralcel OC and Chiralcel OD columns (Diacel) (202). Resolution was improved by hydrogenation. The pentafluorobenzyl ester derivatives of the hydrogenated vicinal diols yielded better separation on the Chiralcel OD column (Diacel, and the elution times were shortened. The *R,R* enantiomers of the dihydroxyeicosanoic acids eluted before the *S,S* enantiomers. It would be useful if these results could be extended to other diols, e.g., to *threo*-17,18-dihydroxyeicosanoic and to *erythro*-17,18-dihydroxyeicosanoic acids.

CONCLUSIONS

PUFA can be oxygenated by P450 in many different ways. Epoxidation, hydroxylation of the ω-side chain, and bisallylic hydroxylation are quantitatively dominant. P450 is a ubiquitous enzyme (2,3). Some of these reactions have been found in other organs and tissues besides liver and kidney cortex. ω-Oxidation was discovered *in vivo* 60 years ago by Verkade *et al.* (203), and it was therefore expected that P450 would oxygenate arachidonic acid *in vivo* in man and in experimental animals.

Biological activities have been associated with many of the P450 metabolites of arachidonic acid. Epoxides of arachidonic acid have attracted the largest interest. Major advances in the last years have been the identification of the constitutive epoxygenases in the human, the rabbit, and the rat kidney. They belong to the CYP2C gene family. Much less is known about the bisallylic hydroxylases. Some of them may belong to the CYP3A family, which is induced by dexamethasone. The metabolic fate and importance of bisallylic hydroxy fatty acids have not yet been explored. The dietary intake of linoleic acid and other PUFA in the human diet is large. It may therefore be of interest to determine whether bisallylic metabolites are formed *in vivo* in humans. In contrast, ω1- and ω2-hydroxylation of saturated fatty acids have been extensively studied in man and many experimental animals. Considering their abundance, the bisallylic hydroxylases of PUFA and their possible biological functions deserve similar attention.

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Mechanism of Linoleic Acid Hydroperoxide Reaction with Alkali

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ABSTRACT: Treatment of (13*S*,9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid (13*S*-HPODE) with strong alkali resulted in the formation of about 75% of the corresponding hydroxy acid, (13*S*,9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoic acid (13*S*-HODE), and the remaining 25% of products was a mixture of several oxidized fatty acids, the majority of which was formed from (9*Z*,11*R,S*,12*S,R*)-13-oxo-11,12-epoxy-9-octadecenoic acid by Favorskii rearrangement (Gardner, H.W., *et al.* (1993) *Lipids 28*, 487–495). In the present work, isotope experiments were completed in order to get further information about the initial steps of the alkali-promoted decomposition of 13*S*-HPODE. **1.** Reaction of [hydroperoxy-$^{18}O_2$]13*S*-HPODE with 5 M KOH resulted in the formation of [hydroxy-^{18}O]13*S*-HODE and [epoxy-^{18}O](9*Z*,11*R,S*,12*S,R*)-13-oxo-11,12-epoxy-9-octadecenoic acid; **2.** treatment of a mixture of [U-^{14}C]13*S*-HODE and [hydroperoxy-$^{18}O_2$]13*S*-HPODE with KOH and analysis of the reaction product by radio-TLC showed that 13*S*-HODE was stable under the reaction conditions and did not serve as precursor of other products; **3.** reaction of a mixture of [U-^{14}C]13-oxo-9,11-octadecadienoic acid (13-OODE) and [hydroperoxy-$^{18}O_2$]13*S*-HPODE with KOH resulted in the formation of [U-^{14}C-epoxy-^{18}O](9*Z*,11*R,S*,12*S,R*)-13-oxo-11,12-epoxy-9-octadecenoic acid; **4.** treatment of a mixture of [hydroperoxy-$^{18}O_2$]13*S*-HPODE and [carboxyl-$^{18}O_1$]13*S*-HPODE with KOH afforded (9*Z*,11*R,S*,12*S,R*)-13-oxo-11,12-epoxy-9-octadecenoic acid having an ^{18}O-labeling pattern which was in agreement with its formation by intermolecular epoxidation. It was concluded that (9*Z*,11*R,S*,12*S,R*)-13-oxo-11,12-epoxy-9-octadecenoic acid is formed from 13*S*-HPODE by a sequence involving initial dehydration into the α,β-unsaturated ketone, 13-OODE, followed by epoxidation of the Δ^{11} double bond of this compound by the peroxyl anion of a second molecule of 13*S*-HPODE. Rapid conversion of hydroperoxides by alkali appeared to require the presence of an α,β-unsaturated ketone intermediate as an oxygen acceptor. This was supported by experiments with a saturated hydroperoxide, methyl 12-hydroperoxyoctadecanoate, which was found to be much more resistant to alkali-promoted conversion than 13*S*-HPODE.
Lipids 31, 1023–1028 (1996).

Frankel *et al.* (1) reported conversion by alkali of methyl linoleate hydroperoxides to their corresponding hydroxy fatty acids without explanation of possible mechanisms of transformation. Recently, this alkali transformation was investigated in more detail showing that (13*S*,9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid (13*S*-HPODE) was converted to (13*S*,9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoic acid (13*S*-HODE) in about 75% yield without loss of regio- and stereoconfiguration (2,3). The majority of remaining 25% of products were proposed (3) to arise from Favorskii Rearrangement of intermediate (9*Z*,11*R,S*,12*S,R*)-13-oxo-11,12-epoxy-9-octadecenoic acid.

O'Brien (4), who reacted a number of "nucleophiles" with linoleic acid hydroperoxide obtained a similar conversion into hydroxyoctadecadienoic acid. Although O'Brien did not test alkali, he suggested that nucleophiles displaced the distal OH from the hydroperoxide group, and this seemed to be a plausible mechanism with the alkali transformation. However, it is difficult to reconcile how the nucleophilic hydroxyl anion could displace the distal hydroperoxide oxygen if the hydroperoxide group exists as a peroxyl anion in strong alkali.

In this communication we investigate the mechanism of alkali transformation of 13*S*-HPODE and compare its reaction with some other hydroperoxides. We show by labeling experiments that hydroperoxide oxygen is consumed by intermolecular epoxidation of a dehydration by-product, (9*Z*,11*E*)13-oxo-9,11-octadecadienoic acid (13-OODE). Evidence is presented to question the possibility of a nucleophilic displacement of the distal hydroperoxide oxygen.

MATERIALS AND METHODS

Materials. Bovine liver catalase 27,000 units/mg was from Sigma (St. Louis, MO). [U-^{14}C] linoleic acid was purchased from Amersham (Buckinghamshire, United Kingdom). $^{18}O_2$ of 99.4 atom % ^{18}O was from Isotec, Inc. (Miamisburg, OH). [Carboxyl-$^{18}O_1$]13*S*-HPODE (5) and [hydroperoxy-$^{18}O_2$]-

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Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; GC–MS, gas chromatography–mass spectroscopy; HPLC, high-performance liquid chromatography; 13*S*-HODE, (13*S*,9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoic acid; 12-HPO, methyl 12-hydroperoxyoctadecanoate; 13*S*-HPODE, (13*S*,9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid; 13-OODE, (9*Z*,11*E*)13-oxo-9,11-octadecadienoic acid; OTMS, trimethylsilyloxy; TLC, thin-layer chromatography; TMS, trimethylsilyl; UV, ultraviolet.

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13*S*-HPODE (6) were prepared as described previously. Methyl 12-hydroperoxyoctadecanoate (12-HPO) was synthesized by the method of Bascetta and Gunstone (7) from methyl 12-hydroxyoctadecanoate from Nu-Chek-Prep (Elysian, MN). 13-OODE was prepared by oxidation of 13*S*-HODE with a CrO3-pyridine complex (8).

Reaction conditions. 13*S*-HPOD (2.3 mM) was treated with 5 M KOH at 35°C, and products were recovered as described previously (3). In certain KOH (always 5 M) treatments, the concentrations of some of the fatty acids or other reactants varied. Reaction of KOH was tested with (i) 0.75 mM uniformly ^{14}C-labeled [U-^{14}C]13*S*-HODE and 5.0 mM [hydroperoxy-$^{18}O_2$]13*S*-HPODE; (ii) 1.0 mM [carboxyl-$^{18}O_1$]13*S*-HPODE and 1.3 mM [hydroperoxy-$^{18}O_2$]13*S*-HPODE; (iii) 0.6 mM [U-^{14}C]13-OODE and 2.3 mM [hydroperoxy-$^{18}O_2$]13*S*-HPODE; (iv) 3.3 mM 12-HPO; and (v) 2.3 mM 12-HPO plus 2.3 mM 13*S*-HPODE.

Other methods. Other methods were essentially as described previously with few exceptions (3). Gas chromatography–mass spectrometry (GC–MS) was accomplished with the same equipment, except selected ion monitoring was utilized to determine isotopic compositions. Developing solvents for thin-layer chromatography (TLC) of methyl esterified products were hexane/ethyl acetate (8:2, vol/vol) and hexane/acetone (92:8, vol/vol). Products of 12-HPO were analyzed as methyl esters by high-performance liquid chromatography (HPLC) with a Microsorb silica column (250 × 4.6 mm, 5 μ spherical particle size; Rainin, Woburn, MA) using a Spectra-Physics (San Jose, CA) SP8800 pump. The isocratic solvent used for 12-HPO products was hexane/isopropanol (191:1, vol/vol) at a flow rate of 0.75 mL/min, and eluants were detected with a Varex (Rockville, MD) ELSD IIA detector (evaporative light-scattering detector) using a detector temperature of 73°C and a gas flow meter setting of 40 mm. Methyl 13*S*-HPODE and methyl 13*S*-HODE were separated with hexane/isopropanol (397:3, vol/vol) solvent at a flow rate of 0.6 mL/min. Detection was absorbance at 235 nm with a Spectra-Physics SP8490 detector.

H_2O_2 measurements. 13*S*-HPODE (385 mM) was incubated with 5 M KOH at 35°C, and 1 mL aliquots were taken at various times. The solution was adjusted to pH 4 with 2 M citric acid, and the aqueous layer was extracted with an equal volume of diethyl ether followed by hexane extraction to remove fatty acids. The extracted fatty acids were saved for analyses of the 13*S*-HODE/13*S*-HPODE ratios. The aqueous layer, containing H_2O_2, was adjusted to pH 6 (3.6 mL total), and a 2.4 mL portion was transferred to an O_2-electrode cell (Gilson Oxygraph, model 5/6H; Middleton, WI). O_2 evolution was measured after addition of 10 μL catalase solution [1 mg bovine liver catalase (27,000 units/mg from Sigma) per mL H_2O].

For determining the peroxide value of the aqueous layer, the same procedure as above was used, except the acidified reaction solution instead was extracted three times with a threefold volume of $CHCl_3$/methanol (2:1, vol/vol) to ensure complete removal of fatty acids in the $CHCl_3$ layer. The peroxide value of the H_2O–methanol layer was determined as described previously (9), except the thiosulfate used in titration was 1×10^{-4} N.

RESULTS AND DISCUSSION

Putative nucleophilic mechanism. One possible mechanism of fatty acid hydroperoxide conversion to fatty acid hydroxides by alkali would be nucleophilic attack at the distal OH of the hydroperoxide group affording fatty acid hydroxyl anion and H_2O_2. This mechanism was proposed by O'Brien (4) for the conversion of hydroperoxide fatty acid into its corresponding hydroxy fatty acid by a number of nucleophiles other than alkali. Previously, our laboratory could not detect a significant amount of H_2O_2 after 3 h of reaction (2). As shown in Table 1, these experiments were extended by (i) using higher concentrations of 13*S*-HPODE (385 mM), (ii) measuring H_2O_2 at a number of shorter times of reaction, and (iii) using two methods of H_2O_2 determination. Under these conditions, H_2O_2 could be detected, but the values were always fairly insignificant. During the first 30 min of reaction, a range of only 0.03–0.33% yield of H_2O_2 based on total 13*S*-HPODE was determined. Either H_2O_2 was a very transient intermediate or it was not formed in significant amounts. Considering alkali as a nucleophilic reagent, a severe constraint would appear to be presented by ionization of the hydroperoxide group to peroxyl anion under these very alkaline conditions, thus preventing attack by hydroxyl anion to form H_2O_2. The acid dissociation constant of the perhydroxyl (hydroperoxide) radical has a pK of 4.88 (10), which should not be much different for organic hydroperoxides. In addition, it was previously shown (2) that O_2 evolution could not be detected during the course of reaction as measured by a War-

TABLE 1
Hydrogen Peroxide Produced During Treatment of (13*S*,9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic Acid (13*S*-HPODE) with 5 N KOH[a]

Reaction time (min)	H_2O_2 generated (mmol/mmol 13*S*-HPODE)		13*S*-HODE/13*S*-HPODE ratio[d]
	O_2-Electrode[b]	Titration[c]	
1	0.0012	0.0033	1:12
15	0.0016	0.0015	1:3.8
30	0.0009	0.0003	1:1.6
45	n.d.	trace	n.d.
60	0	n.d.	1:0.4

[a]13*S*-HPODE (385 mM) was reacted with 5 N KOH terminating the reaction at the times indicated by acidifying to pH 4, extracting the fatty acids, and then analyzing H_2O_2 in the aqueous layer as described in the Materials and Methods section; n.d., not determined.

[b]H_2O_2 was measured by determination of the O_2 released by O_2-electrode after treatment with catalase.

[c]H_2O_2 was measured by determination of peroxide value of the extracted aqueous layer by thiosulfate titration of I_2 released after treatment with acidic KI.

[d]The 13*S*-HODE/13*S*-HPODE ratio of extracted fatty acids was determined by high-performance liquid chromatography separation using ultraviolet detection at 232 nm. 13*S*-HODE, (13*S*,9*Z*,11*E*)-13-hydroxy-9,11-octadecenoic acid.

burg respirometer (a small amount of O_2 was consumed, 11% on a per mole basis). The lack of O_2 evolution was confirmed in this study by the inability to detect $^{18}O_2$ from the headspace of KOH treated [hydroperoxy-$^{18}O_2$]13*S*-HPODE by mass spectral analysis (data not shown). These data together suggest that nucleophilic displacement or O_2 elimination are probably not significant mechanisms.

Disposition of peroxide oxygen. A reaction mixture of [U-^{14}C]13*S*-HODE (0.75 mM) and [hydroperoxy-$^{18}O_2$]13*S*-HPODE (5 mM) in 5 M KOH at 35°C was sampled at 30, 60, and 90 min. The methyl esterified products were separated by TLC with hexane/ethyl acetate (4:1, vol/vol) and radioscanned. Samples obtained at each time showed a single radiolabeled peak due to the methyl ester of [U-^{14}C]13*S*-HODE. Spraying the plate with 8-anitino-1-naphthalenesulfonic acid (ANS) showed methyl esterified (9*Z*,11*R*,*S*,12*S*,*R*)-13-oxo-11,12-epoxy-9-octadecenoic acid and other products unlabeled with ^{14}C. This experiment showed that, once formed from 13*S*-HPODE, 13*S*-HODE is remarkably stable in alkali and that (9*Z*,11*R*,*S*,12*S*,*R*)-13-oxo-11,12-epoxy-9-octadecenoic acid and other by-products do not originate from 13*S*-HODE.

Reaction of [hydroperoxy-$^{18}O_2$]13*S*-HPODE (98+% isotopic purity) with 5 M KOH showed that product 13*S*-HODE retained one ^{18}O as expected as determined by the mass spectrum of its methyl ester/13-trimethylsilyloxy (OTMS) derivative: [*m/z* (percentage relative intensity, ion structure)] 384 (22, M^+); 313 (50, $[M - CH_3(CH_2)_4]^+$); 227 (23, $[M - (CH_2)_7COOCH_3]^+$); 73 [100, trimethylsilyl (TMS^+)]. Of significance to the mechanism, there was ^{18}O transfer to the other products. The important product (9*Z*,11*R*,*S*,12*S*,*R*)-13-oxo-11,12-epoxy-9-octadecenoic acid, intermediate to other compounds, was ^{18}O-labeled at the 11,12-epoxide. The 13-ketone was completely devoid of ^{18}O-label, presumably due to exchange with water. Compared with the same unlabeled fatty acid methyl ester (3), the important features of its mass spectrum were: 326 (28, M^+); 306 (28, $[M - H_2{}^{18}O]^+$); 295 (22, $[M - CH_3O]^+$); 266 (12); 223 (21, $[M - CH_3(CH_2)_4 - CH_3OH]^+$); 178 (61); 169 (43, $[M - (CH_2)_7COOCH_3]^+$); 140 (53); 99 (86, $CH_3(CH_2)_4CO^+$). The ^{18}O-labeling of Favorskii Rearrangement products derived from (9*Z*,11*R*,*S*,12*S*,*R*)-13-oxo-11,12-epoxy-9-octadecenoic acid, that is, 2-butyl-4-hydroxy-5-tetradecendioic acid and 2-butyl-3,5-tetradecadiendioic acid, were also examined by GC–MS. The mass spectrum of 2-butyl-3,5-tetradecadiendioic acid, dimethyl ester, was indistinguishable from the unlabeled compound previously reported (3) in agreement with its origin from the oxoepoxyene fatty acid with loss of the ^{18}O-labeled 11,12-epoxide. There are two isomers of product 2-butyl-4-hydroxy-5-tetradecendioic acid. One isomer (determined as its dimethyl ester/OTMS derivative) contained one ^{18}O-label as indicated by its *m/z* ion 398 (6, $[M - CH_3OH]^+$). Compared with its previously determined spectrum (3), the label was certainly not located on the most highly substituted carboxylate group as indicated by its *m/z* 371 (9, $[M - COOCH_3]^+$), but it was probably located at the 4-hydroxyl as indicated by its *m/z* 287 (100, $TMS^{18}OCH{-}CH{=}CH(CH_2)_7COOCH_3^+$). This result is in agreement with its origin from (9*Z*,11*R*,*S*,12*S*,*R*)-13-oxo-11,12-epoxy-9-octadecenoic acid with retention of the epoxide oxygen as a 4-hydroxyl. In contrast, the other isomer of 2-butyl-4-hydroxy-5-tetradecendioic acid had lost much of its ^{18}O-label as indicated by its *m/z* 285/287 ratio of 1:0.65. Evidently, this latter isomer may be more susceptible to forming the lactone observed previously (3), thus permitting exchange of oxygen. Also, the product, 11-hydroxy-9,12-heptadecadienoic acid (3), was labeled with one ^{18}O. Prominent features of its mass spectrum (methyl ester/OTMS derivative) were: 370 (23, M^+); 313 (25, $[M - CH_3(CH_2)_3]^+$); 213 (46, $[M - (CH_2)_7COOCH_3]^+$); 73 (100, TMS^+).

*Precursor to formation of (9Z,11*R,S,*12*S,R*)-13-oxo-11,12-epoxy-9-octadecenoic acid.* 13-OODE was a consistent, but low-yield, product in reactions of 13*S*-HPODE with alkali (3). It was postulated that this ketodiene fatty acid could be generated from 13*S*-HPODE by dehydration and subsequently act as an acceptor of peroxide oxygen to form (9*Z*,11*R*,*S*,12*S*,*R*)-13-oxo-11,12-epoxy-9-octadecenoic acid. This hypothesis was tested by reacting [U-^{14}C]13-OODE (0.6 mM) and [hydroperoxy-$^{18}O_2$]13*S*-HPODE (2.3 mM) with 5 M KOH at 35°C taking aliquot samples at 1 and 40 min. The esterified products were separated by TLC with hexane/acetone (92:8, vol/vol) solvent. Radioscanning of the 1-min sample showed one radiolabeled band due to unconverted [U-^{14}C]13-OODE. After spraying with ANS spray no fluorescent band due to the oxoepoxyene fatty ester was visible under ultraviolet (UV) light, but unconverted 13-OODE could be seen as a UV absorbing band. The 40-min products separated into a strongly radiolabeled band due to methyl (9*Z*,11*R*,*S*,12*S*,*R*)-13-oxo-11,12-epoxy-9-octadecenoate (48,600 c.p.m.) and a faintly labeled band due to [U-^{14}C]13-OODE (2,944 c.p.m.). ANS spray and UV viewing revealed the two bands as the oxoepoxyene fatty ester (fluorescent) and 13-OODE (UV absorbing). GC–MS analysis showed the same prominent ions as described above for (9*Z*,11*R*,*S*,12*S*,*R*)-13-oxo-11,12-epoxy-9-octadecenoic acid derived from [hydroperoxy-$^{18}O_2$]13*S*-HPODE alone showing that it was labeled with one ^{18}O located at the 11,12-epoxide. This oxoepoxyene fatty acid was also generated from 13-OODE simply by incubation with H_2O_2 and 5 M KOH (data not shown). Therefore, (9*Z*,11*R*,*S*,12*S*,*R*)-13-oxo-11,12-epoxy-9-octadecenoic acid (OEOE in equation below) can be formed by epoxidation of 13-OODE in the presence of either 13*S*-HPODE or H_2O_2 and 5 M KOH as indicated by Equation 1:

$$\text{13-OODE} + \text{13}S\text{-HPODE (or } H_2O_2) + \text{KOH} \rightarrow \text{OEOE} + \text{13}S\text{-HODE (or } H_2O) + \text{KOH} \quad [1]$$

*Mechanism of oxygen transfer to (9Z,11*R,S,*12*S,R*)-13-oxo-11,12-epoxy-9-octadecenoic acid.* Intermolecular transfer of peroxide oxygen to the 11,12-double bond of 13-OODE in alkali is a reasonable assumption. This possibility was tested by reaction of [carboxyl-$^{18}O_1$]13*S*-HPODE (1.0 mM)

TABLE 2
Intermolecular Transfer of Hydroperoxide Oxygen to the 11,12-Epoxide of (9*Z*,11*R*,*S*,12*S*,*R*)-13-Oxo-11,12-epoxy-9-octadecenoic acid (OEOE)

Compound analyzed (as derivatives)	Isotopic composition
13*S*-HPODE reactant[a]	9% $^{18}O_0$; 42% carboxyl $^{18}O_1$; 49% hydroperoxide $^{18}O_2$
13*S*-HPODE recovered	10% $^{18}O_0$; 40% carboxyl $^{18}O_1$; 50% hydroperoxide $^{18}O_2$
13S-HODE	10% $^{18}O_0$; 42% carboxyl $^{18}O_1$; 48% hydroxy $^{18}O_1$
13-Oxo-9,11-octadecadienoate	23% $^{18}O_0$; 43% carboxyl $^{18}O_1$; 34% keto $^{18}O_1$
OEOE[b], found	29% $^{18}O_0$; 47% $^{18}O_1$; 24% $^{18}O_2$
OEOE, intermolecular reaction, calculated	29% $^{18}O_0$; 50% $^{18}O_1$; 21% $^{18}O_2$
OEOE, intramolecular reaction, calculated	9% $^{18}O_0$; 91% $^{18}O_1$; 0% $^{18}O_2$

[a]1 mM of [carboxyl-$^{18}O_1$]13*S*-HPODE (96% $^{18}O_1$; 4% $^{18}O_0$) and 1.3 mM [hydroperoxy-$^{18}O_2$]13*S*-HPODE (87% $^{18}O_2$; 13% $^{18}O_0$) was reacted with 5 M KOH at 35°C for 40 min. See Table 1 for other abbreviations.
[b]Under the conditions used, any ^{18}O-label at the C-13 keto oxygen of OEOE would have been lost because of exchange with water. This was proved in a separate experiment in which OEOE produced from [hydroperoxy-$^{18}O_2$]13*S*-HPODE was found to contain ^{18}O-label at the epoxide oxygen, but not at the keto oxygen.

and [hydroperoxy-$^{18}O_2$]13*S*-HPODE (1.3 mM) with 5 M KOH at 35°C for 40 min. The methyl esters of 13*S*-HODE, 13-OODE, and (9*Z*,11*R*,*S*,12*S*,*R*)-13-oxo-11,12-epoxy-9-octadecenoic acid were isolated by TLC after development with hexane/acetone (92:8, vol/vol). The isolates were analyzed by GC–MS using selected ion monitoring furnishing results consistent with intermolecular epoxidation in the formation of the oxoepoxyene fatty acid (Table 2).

Stability of saturated hydroperoxides. The availability of an activated double bond of 13-OODE seemed necessary in order to "reduce" 13*S*-HPODE into 13*S*-HODE. Thus, the fully saturated methyl 12-HPO was reacted with 5 M KOH. As expected, this hydroperoxide was resistant to alkali (Table 3). With extended times of incubation, 12-HPO was converted mainly into 12-oxooctadecanoic acid with only small amounts of 12-hydroxyoctadecanoic acid. 12-Oxooctadecanoic acid (methyl ester) was identified by its mass spectrum as follows: [*m/z* (percentage relative intensity, ion structure)] 312 (1.4, M^+); 281 (17, $M^+ - CH_3O$); 242 (22, $^+CH_2{=}COH(CH_2)_{10}COOCH_3$); 227 (17, $^+CO(CH_2)_{10}COOCH_3$); 185 (20); 167 (15); 153 (40); 128 (61, $CH_3(CH_2)_5COH{=}CH_2^+$); 113 (73, $CH_3(CH_2)_5CO^+$); 98 (80); 85 (57); 69 (70); 55 (100). 12-Hydroxyoctadecanoic acid was identified by its mass spectrum as its methyl ester trimethylsilyloxy derivative: [*m/z* (percentage relative intensity, ion structure)] 355 (2.4, $M^+ - CH_3O$); 339 (7, $M^+ - CH_3OH - CH_3$); 301 (59, $M+ - CH_3(CH_2)_5$); 187 (100, $CH_3(CH_2)_5CHOTMS^+$); 73 (49, TMS).

When 13*S*-HPODE was also included in the reaction with 12-HPO (Table 3), the conversion of 12-HPO was more rapid implying that the unsaturation provided by 13*S*-HPODE was responsible.

A proposed mechanism and other considerations. As shown in Figure 1, a mechanism is proposed that is consistent with the results. We propose that the first step is dehydration of 13*S*-HPODE to afford 13-OODE. Although 13-OODE is an intermediate that does not accumulate, 12-HPO in the presence of alkali was converted mainly to 12-oxooctadecanoic acid, inferring that conversion to the ketone is an important reaction with 13*S*-HPODE also. This ketodiene fatty acid provides the labile double bond that permits transfer of one distal oxygen from the peroxyl anion of 13*S*-HPODE to the 11,12-epoxide of (9*Z*,11*R*,*S*,12*S*,*R*)-13-oxo-11,12-epoxy-9-octadecenoic acid. As discussed previously (3), this oxoepoxyene fatty acid evidently undergoes Favorskii Rearrangement through an unobserved cyclopropanone intermediate to furnish 2-butyl-4-hydroxy-5-tetradecendioic acid and 2-butyl-3,5-tetradecadiendioic acid. It seems plausible that

TABLE 3
Conversion of Methyl 12-Hydroperoxyoctadecanoate (12-HPO) by Alkali with and without 13*S*-HPODE[a]

	12-HPO alone			12-HPO + 13*S*-HPODE		
Time (h)	Unreacted (mg)	12-HO[b] (mg)	12-OO[b] (mg)	Unreacted 12-HPO (mg)	Unreacted 13*S*-HPODE (mg)	12-HO + 13-HODE[c] (mg)
0	1.07	0.01	0	0.77	0.72	0.01
1	0.68	0.01	0.39	0.28	0.12	0.12
4	0.43	0.03	0.30			
6				0.10	0.02	0.31
12	0.39	0.04	0.45			

[a]The reaction was always 5 M KOH at 35°C. Incubations with 12-HPO alone was with 3.3 mM 12-HPO (1.09 mg 12-HPO/mL); with 12-HPO plus 13*S*-HPODE the concentration of both was 2.3 mM (0.76 mg 12-HPO/mL and 0.72 mg 13*S*-HPODE/mL). Material from 1 mL aliquots was analyzed as methyl esters. See Table 1 for other abbreviations.
[b]12-HO, methyl 12-hydroxyoctadecanoate; 12-OO, methyl 12-oxooctadecanoate.
[c]Methyl 12-hydroxyoctadecanoate and 13-HODE could not be separated by high-performance liquid chromatography, and thus, are tabulated together as a sum.

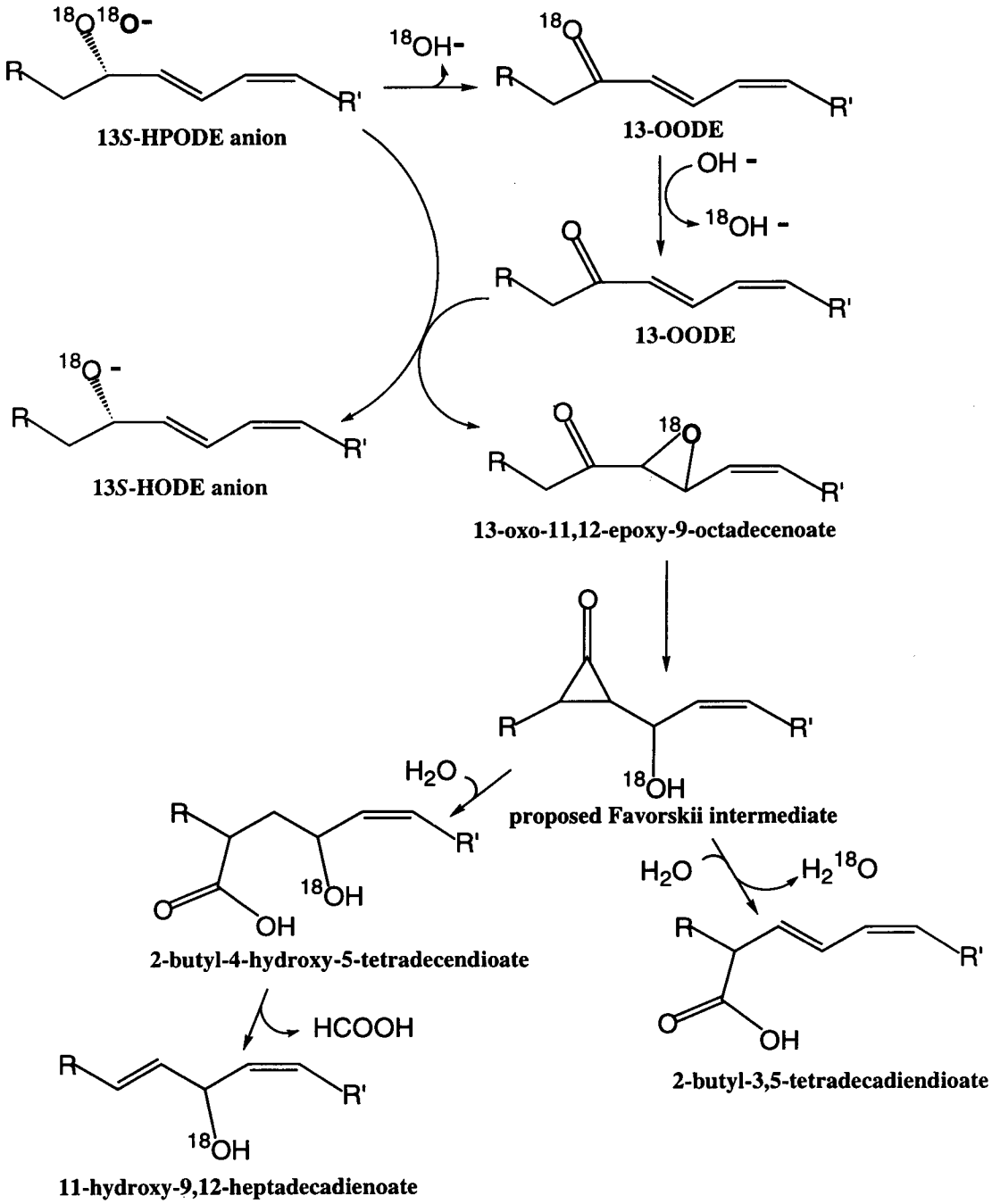


FIG. 1. Proposed reaction mechanism of (13*S*,9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoate (13*S*-HPODE) by alkali into (13*S*,9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoate (13*S*-HODE) and other fatty acids (as their K salts). 13-OODE, (9*Z*,11*E*)-13-oxo-9,11-octadecadienoate; R = $CH_3(CH_2)_3$-; R′ = $-(CH_2)_7COO^-K^+$; the bold ^{18}**O** indicates one oxygen transfer from hydroperoxy group to the epoxy group.

11-hydroxy-9,12-heptadecadienoic acid could arise from 2-butyl-4-hydroxy-5-tetradecendioic acid by elimination of formic acid, but the only evidence for this eventuality was a consistent ^{18}O-labeling pattern of the hydroxyl group.

Although the mechanism would seem to be completely assessed by our experiments, the stoichiometry of product formation is not balanced. If one considers that "dehydration" to 13-OODE is the origin of 25% of products other than 13*S*-HODE, then only 25% of the total serves as a sink for peroxide oxygen. Taken to completion, this would result in a 1:1 ratio of 13*S*-HODE to other products; whereas, the actual ratio was 3:1 (3). Obviously, there appear to be oxygen consumptive reactions unknown to us. For example, a number of minor products were short-chain dienedioc and monoenedioic acids, such as 3-hydroxy-4-tridecenedioic acid (3), and these compounds could arise from secondary oxidations not shown

in Figure 1. However, the minor quantity of short-chain compounds found gives reason to doubt that these potential oxidations would entirely account for the stoichiometric imbalance. One possibility is an oxygen consumption in the "dehydration" of 13*S*-HPODE to 13-OODE. A reaction incorporating such a feature is known as the "Russell Mechanism" which combines two peroxyl radicals of 13*S*-HPODE, yielding a molecule each of 13*S*-HODE and 13-OODE, as well as singlet oxygen (11). This would lead to a more realistic 2:1 ratio of 13*S*-HODE to other products. Evidentally, this reaction is not occurring because neither oxygen nor products of reaction between 13*S*-HODE and singlet oxygen were observed when [U-^{14}C]13*S*-HODE was incubated with 13*S*-HPODE.

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Effects of Membrane Charges and Hydroperoxides on Fe(II)-Supported Lipid Peroxidation in Liposomes

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ABSTRACT: The processes in producing a lag phase in Fe^{2+}-supported lipid peroxidation in liposomes were investigated. Incorporation of phosphatidylserine (PS) or dicetyl phosphate (DCP) into phosphatidylcholine [PC(A)] liposomes, which have arachidonic acid, produced a marked lag phase in Fe^{2+}-supported peroxidation, where PS was more effective than DCP. Phosphatidylcholine dipalmitoyl [PC(DP)] with a net-neutral charge was still effective in producing a lag phase, though weak. Increasing concentrations of PS, DCP, and PC(DP) prolonged the lag period. Initially after adding Fe^{2+}, slight oxygen consumption occurred in PC(A)/PS liposomes including hydroperoxides, followed by a lag phase. An increase in the hydroperoxide resulted in a shortening of the lag period. The initial events of Fe^{2+} oxidation accompanied by oxygen consumption were dependent on the hydroperoxide content, but significant changes in diene conjugation and hydroperoxide levels at this stage were not found. The molar ratios of both disappeared Fe^{2+} and consumed O_2 to preformed hydroperoxide in liposomes with or without *tert*-butylhydroxytoluene were constant, regardless of the different amounts of lipid hydroperoxides. The antioxidant completely inhibited the propagation of lipid peroxidation in the lipid phase, following a lag phase. In a model system containing 2,2′-azobis (2-amidinopropane) dihydrochloride, Fe^{2+} were consumed. We suggest that Fe^{2+} retained at a high level on membrane surfaces play a role in producing a lag phase following the terminating behavior of a sequence of free radical reactions initiated by hydroperoxide decomposition, probably by intercepting peroxyl radicals.
Lipids 31, 1029–1038 (1996).

Lipid peroxidation in biomembranes is mediated by free radical reactions, including peroxidative fragmentation of phospholipids, leading to degeneration of the membranes associated with a variety of pathological events. Iron is known as an effective catalyst of lipid peroxidation (1). Iron-catalyzed lipid peroxidation is affected by the surface charges of membranes. For instance, Kunimoto *et al.* (2) observed that neutrally or negatively charged liposomes are more sensitive to ferrous ascorbate-supported peroxidation than to positively charged liposomes, and Fukuzawa *et al.* (3) showed that Fe^{2+}-catalyzed lipid peroxidation of linoleic acid in the presence of hydroperoxides was more effective in negatively charged micelles than in positively charged micelles. The Fe^{2+} complex is also effective in promoting lipid peroxidation of negatively charged liposomes in a nonenzymatic system (4) or in a reconstituted enzymatic system (5).

However, adding Fe^{2+} to mitochondria (6), microsomes (7,8), or phospholipid liposomes (2,9,10) produces a lag phase before the maximal rates of lipid peroxidation. The lag period is lengthened with an increase in added Fe^{2+}, and is shortened or eliminated by the coexistence of ferric iron (1,10), divalent, or trivalent metals without a redox capacity (8–10), or spermine (11). Alternatively, Yoshida *et al.* (12) reported that phosphatidylserine incorporated into phosphatidylcholine liposomes inhibits lipid peroxidation induced by ferrous-ascorbate. Similarly, Kogure *et al.* (11) showed that egg yolk phosphatidylcholine/bovine heart cardiolipin liposomes are resistant to ADP/Fe^{2+}-induced lipid peroxidation. These observations suggest that the net-negative surface charges of membranes in the appearance of the lag phase in lipid peroxidation may be important, in which case the lag phase could be produced by increased accumulation of Fe^{2+} on membranes (9). However, Fe^{2+}-supported lipid peroxidation in phosphatidylcholine liposomes also produces a lag phase (10), but it is unclear why the liposomes with the net-neutral charge are responsible for producing the lag phase.

Microsomes always contain traces of preformed lipid hydroperoxides (8). When liposomes are prepared by sonication, generation of traces of lipid peroxides is inevitable (8,13). Thus, obtaining peroxide-free lipid systems in biochemical experiments is difficult (8,14). The Fe^{2+} added to lipid systems with preformed hydroperoxides stimulate lipid peroxidation by decomposing hydroperoxides to free radicals leading to propagation reaction. Driomina *et al.* (9) observed that on adding Fe^{2+} an initial rise in malondialdehyde concentration before a lag phase was apparently associated with the decomposition of lipid peroxides. If alkoxyl and peroxyl radicals are generated during the process, the lag phase might reflect the duration of inhibition of lipid peroxidation.

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Abbreviations: AAPH, 2,2′-azobis (2-amidinopropane) dihydrochloride; BPS, bathophenanthroline disulfonic acid disodium; BHT, *tert*-butylhydroxytoluene; DCP, dicetyl phosphate; PC(A), L-α-phosphatidylcholine β-arachidonoyl γ-stearoyl; PC(DP), L-α-phosphatidylcholine dipalmitoyl; PS, DL-α-phosphatidyl-L-serine dipalmitoyl; TBARS, thiobarbituric acid-reactive substances.

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The effect of membrane charge, as well as the behavior of Fe^{2+}, on producing a lag phase in lipid peroxidation is thus poorly understood; especially unclear are the molecular events in the processes leading up to propagation reaction. In this study, to clarify the mechanism producing a lag phase in Fe^{2+}-supported lipid peroxidation, we examined the effects of different surface charges of liposomes on the length of the lag phase and the chemical changes in the early stages before the lag phase, using phosphatidylcholine liposomes with different levels of lipid hydroperoxides. We show (i) the importance of anions on membrane surfaces in producing a lag phase, and (ii) the occurrence of Fe^{2+} oxidation accompanied by oxygen consumption in the early stages, is caused through free radical reactions, including hydroperoxide decomposition. We discuss the production of a lag phase in relation to redox cycling of iron on the membrane surfaces.

MATERIALS AND METHODS

Materials. L-α-phosphatidylcholine β-arachidonoyl γ-stearoyl [PC(A)], DL-α-phosphatidyl-L-serine dipalmitoyl (PS), L-α-phosphatidylcholine dipalmitoyl [PC(DP)], and dicetyl phosphate (DCP) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of commercial reagent grade quality. Water processed with a Milli-Q system (Millipore, Milford, MA) was used to prepare all reagents. Ferrous ions were used as $FeSO_4$, dissolved carefully to avoid autoxidation in water purged with argon, and the solution (1.5 mM $FeSO_4$, pH 6.0) was kept under argon during the experiments. Under these conditions, no iron oxidation occurred. In this study, $FeSO_4$ below 150 μM was added directly to the reaction medium, including 25 mM Tris-HCl buffer at pH 7.5, as it produced no change in the pH of the buffer.

Preparation of lipid hydroperoxide. A solution of 100 mg PC(A) in 10 mL of chloroform/methanol (1:1, vol/vol) containing 5 mg methylene blue as a photosensitizer was placed in a beaker (diameter, 7 cm) and was stirred gently under photoirradiation for 3 h at 10°C. The light source, a 100 W tungsten proinjection lamp, was held at a 30-cm distance above the surface of the reaction mixture. The reaction mixture was passed through a column packed with Florisil (100–200 mesh) (Wako Chemicals, Tokyo, Japan) to remove methylene blue. The column was then washed with chloroform/methanol (1:2, vol/vol) followed by washing with methanol. After removing the solvents under argon, the lipids containing hydroperoxides were dissolved in chloroform. The hydroperoxide was determined by the method of Buege and Aust (15). Phosphorus in the lipids following oxidative degradation was estimated by the method of Fiske and Subbarow (16).

Preparation of liposomes. The PC(A), which has arachidonic acid at the β-position, was used as the substrate of lipid peroxidation, and PC(A) liposomes with different surface charges were prepared. A solution of 3.8 μmol of PC(A) and 0.38–1.15 μmol of PS, PC(DP), or DCP in chloroform were added to a small bottle, the chloroform was removed under argon, and the bottle was purged with argon after adding 1 mL of 0.1 M Tris-HCl at pH 7.5 containing 0.25 M NaCl, and then sealed. Unilamellar liposomes were prepared by sonication as we described previously (4). All operations were carried out under argon, and sonication was performed in ice-cold water. The hydroperoxide content of PC(A) in the liposomes after sonication was 2.9 ± 0.3 mol% (n = 20). Liposomes including hydroperoxides at higher concentrations were prepared as described above in this section, by adding different amounts of preformed hydroperoxides prepared by photoirradiation to PC(A) and PS dissolved in chloroform. When necessary, *tert*-butylhydroxytoluene (BHT) was also incorporated into the liposomes. The hydroperoxide levels of these liposomes after sonication were estimated and expressed as a liposomal concentration.

The stability of differently charged liposomes was compared by 5(6)-carboxyfluorescein- leakage (17). The proportion of the dye-leakage from 0.1 mM PC(A) liposomes after incubation in 0.15 M KCl and 25 mM Tris-HCl buffer (pH 7.4) at 37°C for 30 min was 6%, and incorporation of PC(DP), PS, or DCP at the concentrations used in this study caused no differences in leakiness. However, 11% releases were found by incorporating 20 μM stearylamine.

Lipid peroxidation. The incubation mixture contained phospholipid liposomes, Fe^{2+}, 0.15 M KCl, and 25 mM Tris-HCl buffer at pH 7.5 in a final volume of 1.5 mL. After 2 min, with a temperature equilibration at 37°C, the reaction was started by adding 10–100 μL of 1.5 mM Fe^{2+}. Thiobarbituric acid-reactive substances (TBARS) were quantified by the method of Ottolenghi (18), using a value of the extinction coefficient at 535 nm of 156 $mM^{-1}cm^{-1}$, and the result was expressed as production of malondialdehyde. The background autoxidative activity (no incubation) of malondialdehyde production was subtracted in each experiment. Oxygen consumption was measured in a 1.5 mL cell at 37°C with a Clark electrode in a YSI Model 5300 Biological Oxygen Monitor (Yellow Springs Instruments, Yellow Springs, OH) and was calibrated by the method of Misra and Fridovich (19).

Assay of iron. The incubation mixture contained phospholipid liposomes, Fe^{2+}, 0.15 M KCl, and 25 mM Tris-HCl buffer at pH 7.5 in a final volume of 1.5 mL. After 2 min, with temperature equilibration at 37°C, the reaction was started by adding 50 or 100 μL of 1.5 mM Fe^{2+}. The concentration of the remaining Fe^{2+} after incubation was determined colorimetrically using bathophenanthroline disulfonic acid disodium (BPS). An aliquot (1.0 mL) of the incubation mixture was added to 0.6 μmol of BPS in a final volume of 2.0 mL, and the absorbance at 534 nm was measured. An extinction coefficient of 22,140 $M^{-1}cm^{-1}$ for Fe^{2+}-BPS was used (20). The Fe^{3+} were monitored by recording an increase in absorbance at 310 nm at 37°C (21).

RESULTS

Figure 1 shows the profiles of Fe^{2+}-supported oxygen consumption in 100 μM PC(A) liposomes with different charges.

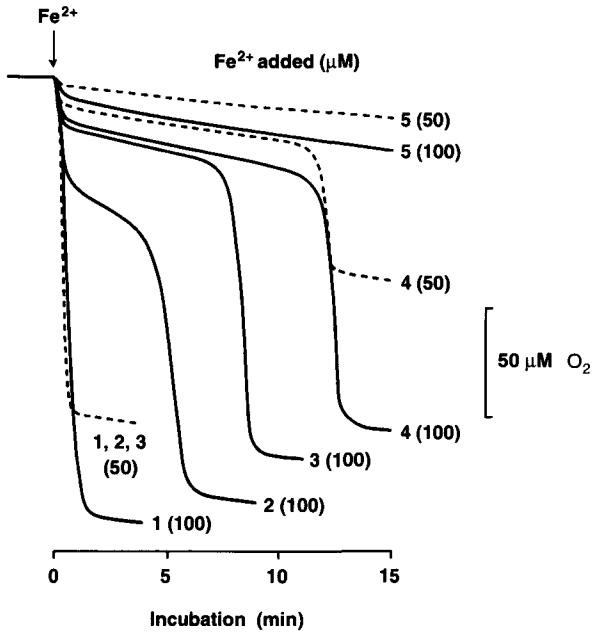


FIG. 1. Fe^{2+}-supported oxygen consumption of L-α-phosphatidylcholine β-arachidonoyl γ-stearoyl [PC(A)] liposomes with different surface charges. Phospholipid liposomes were prepared as described in the Materials and Methods section. The incubation mixtures contained 0.1 mM PC(A) liposomes, alone or including 20 μM of L-α-phosphatidylcholine dipalmitoyl [PC(DP)], dicetyl phosphate (DCP), or DL-α-phosphatidyl-L-serine dipalmitoyl (PS), 50 or 100 μM Fe^{2+}, 0.15 M KCl, and 25 mM Tris-HCl buffer at pH 7.5. Oxygen consumption was monitored as described in the Materials and Methods section. The dotted lines indicate 50 μM Fe^{2+}, and the solid lines indicate 100 μM Fe^{2+}. 1, PC(A) alone; 2, PC(A)/PC(DP); 3, PC(A)/DCP; 4, PC(A)/PS; 5, without liposomes (Fe^{2+} autoxidation). The addition of Fe^{2+} is indicated by an arrow. This set of oxygraphs was one of three separate sets of experiments which showed essentially the same pattern.

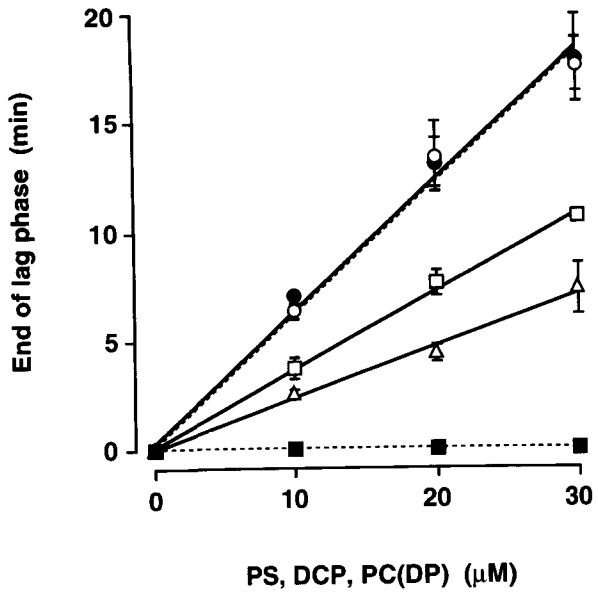


FIG. 2. Duration of the lag time of Fe^{2+}-supported oxygen consumption in PC(A) liposomes with different surface charges. Phospholipid liposomes were prepared as described in the Materials and Methods section. The composition of incubation mixtures is the same as in Figure 1, except that the liposomes, including PS, DCP, or PC(DP), at the indicated concentrations were used. The solid lines with open symbols indicate 100 μM Fe^{2+} addition: ○, PS; □, DCP; △, PC(DP), and the dotted lines with closed symbols indicate 50 μM Fe^{2+} addition: ●, PS; ■, DCP or PC(DP). Data represent the means ± SD of triplicate determinations. See Figure 1 for abbreviations.

With 50 μM Fe^{2+}, maximal rates of oxygen consumption occurred immediately after adding Fe^{2+} to PC(A) liposomes, and similarly, also in PC(A) liposomes incorporating either PC(DP) with a net-neutral charge or DCP with a negative charge. In contrast, in PC(A) liposomes containing PS with a net-negative charge, a small amount of oxygen was consumed initially after adding Fe^{2+}, followed by a period of slow oxygen consumption (lag phase), and, finally, a large amount of oxygen was quickly consumed. The same duration of lag phase was obtained with 100 μM Fe^{2+}, but a higher amount of oxygen was consumed after the lag. Similarly, in PC(A) liposomes containing PC(DP) or DCP, adding 100 μM Fe^{2+} produced a lag phase in oxygen consumption, but the length of the lag phase was different between both liposomes. It is noteworthy that a slight drop in each curve of oxygen consumption, in comparison with Fe^{2+} autoxidation, was always observed before the appearance of the lag phase. The lag periods of Fe^{2+}-supported oxygen consumption in PC(A) liposomes with PS, DCP, or PC(DP) at different concentrations were compared (Fig. 2). In all cases, except for DCP or PC(DP) with 50 μM Fe^{2+}, the lag periods lengthened linearly as a function of the concentrations of PS, DCP, or PC(DP). The ability of these compounds to prolong the lag period was in the order of PS>DCP>PC(DP). In PC(A) liposomes containing PS, the length of lag period at various concentrations of Fe^{2+} and the total oxygen consumption after the lag phase were estimated (Fig. 3). At all concentrations of Fe^{2+}, an increase in PS concentration lengthened the lag period. The length of the lag phase increased with increase in concentration of Fe^{2+} added up to 40 μM, but was constant above this concentration, which may reflect that the membrane surfaces are possibly saturated with Fe^{2+} at higher concentrations. At any concentration of PS, the total oxygen consumption after the lag phase constantly increased with an increase in Fe^{2+} concentrations, whereas the higher the concentration of PS, the lower was the oxygen consumption.

The changes in iron concentrations during Fe^{2+}-supported lipid peroxidation were monitored (Fig. 4). Generally, the oxygen consumption in either PC(A) liposomes or PC(A)/PS liposomes was accompanied by TBARS production, and these parameters correlated well with Fe^{2+} disappearance and Fe^{3+} formation in all processes of Fe^{2+}-supported lipid peroxidation, except that the initial consumption of oxygen in PC(A)/PS liposomes was not accompanied by TBARS production (the same level as without incubation). The rates of Fe^{2+} disappearance and Fe^{3+} formation during the lag phase

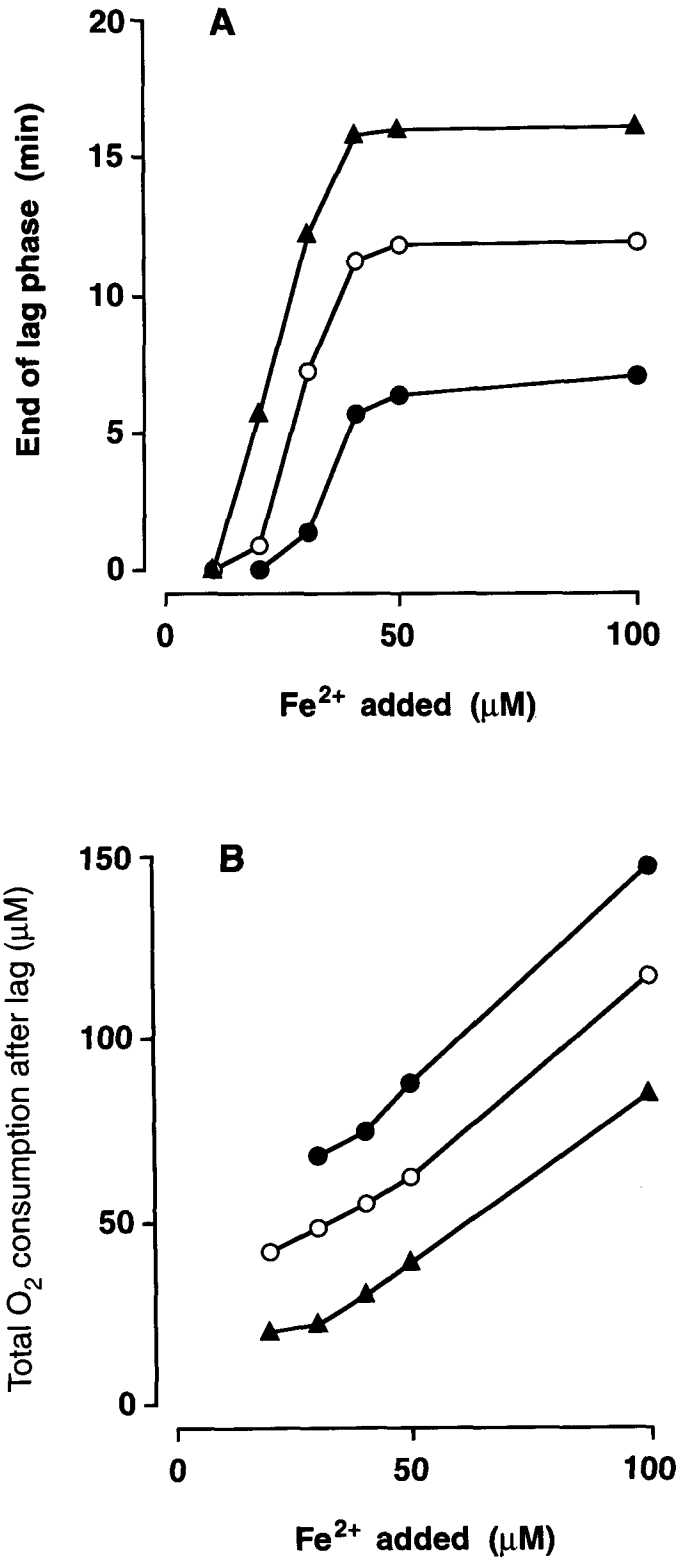


FIG. 3. Effects of Fe^{2+} at different concentrations on duration of lag time (A) and total oxygen consumption after lag (B) in PC(A)/PS liposomes. The composition of incubation mixtures is the same as in Figure 2, except that Fe^{2+} at the indicated concentrations was added. ●, 10 μM PS; ○, 20 μM PS; ▲, 30 μM PS. These data were one of two separate sets of experiments which showed essentially the same pattern. See Figure 1 for abbreviations.

were little changed compared with Fe^{2+} autoxidation without liposomes. After the lag phase, Fe^{2+} instantaneously disappeared with lipid peroxidation.

The effect of different levels of lipid hydroperoxides on oxygen consumption and Fe^{2+} oxidation in PC(A)/PS liposomes was examined (Fig. 5). All oxygen consumption curves in these systems showed a good correlation with Fe^{2+} oxidation. The lag periods in both parameters shortened with increased amounts of lipid hydroperoxides in the liposomes (lines 1 and 2), but lengthened with increased amounts of added Fe^{2+} (lines 2 and 3). The initial oxygen consumption and the accompanying oxidation of Fe^{2+} were affected by the hydroperoxide levels in liposomes (lines 1 and 2). Figure 6 shows that the lag periods in oxygen consumption in the presence of 50 μM or 100 μM Fe^{2+} varied inversely as the hydroperoxide content in the liposomes, and no lag period in the presence of 50 μM Fe^{2+} was produced at higher than the indicated hydroperoxide content. These results showed that the shortening of the lag phase is caused by preformed hydroperoxides *via* oxidation of Fe^{2+}.

In the early stages in Fe^{2+}-supported systems with preformed hydroperoxides, significant changes in the absorbance due to diene conjugation and the peroxide level were not found, while after the lag phase, rapid increases in these parameters reflected the propagation of lipid peroxidation (Fig. 7). The effect of BHT on Fe^{2+}-supported lipid peroxidation was examined (Fig. 8). Initially after adding Fe^{2+}, both oxygen consumption and Fe^{2+} oxidation between liposomes with or without BHT showed no differences, whereas BHT completely inhibited both lipid peroxidation and Fe^{2+} oxidation after the lag phase. In the early stages, Fe^{2+} disappeared and oxygen consumed were determined 1 min after incubation, using liposomes with different lipid hydroperoxide contents with or without BHT (Fig. 9). The disappearance of Fe^{2+} in the presence of 100 μM Fe^{2+} increased with increase in preformed hydroperoxides. No significant difference in Fe^{2+} disappearance was found between liposomes with or without BHT when two regression slopes or intercepts were compared by the Student's *t*-test. The molar ratio of disappeared Fe^{2+}/preformed hydroperoxide included in the liposomes was approximately three, while under argon without BHT, the ratio was two. Similarly, oxygen consumption depended on the content of preformed hydroperoxides, and BHT showed no significant influence on the oxygen consumption. About 2.5–2.8 moles of oxygen to a preformed hydroperoxide were consumed.

Coassin *et al.* (22) reported that Fe^{2+} is ineffective to inhibit crocin bleaching by peroxyl radicals, while iron is rapidly oxidized. The reactivity of Fe^{2+} with free radicals was examined in a model system containing 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) (Fig. 10), which thermally decomposes to give two 2-amidinopropane radicals (A•), either recombining in a solvent cage or coupling with oxygen to give peroxyl radicals (23). In our study, Fe^{2+} were consumed depending on the amounts of AAPH under air (r = 0.995) or under argon (r = 0.988), but the consumption rate

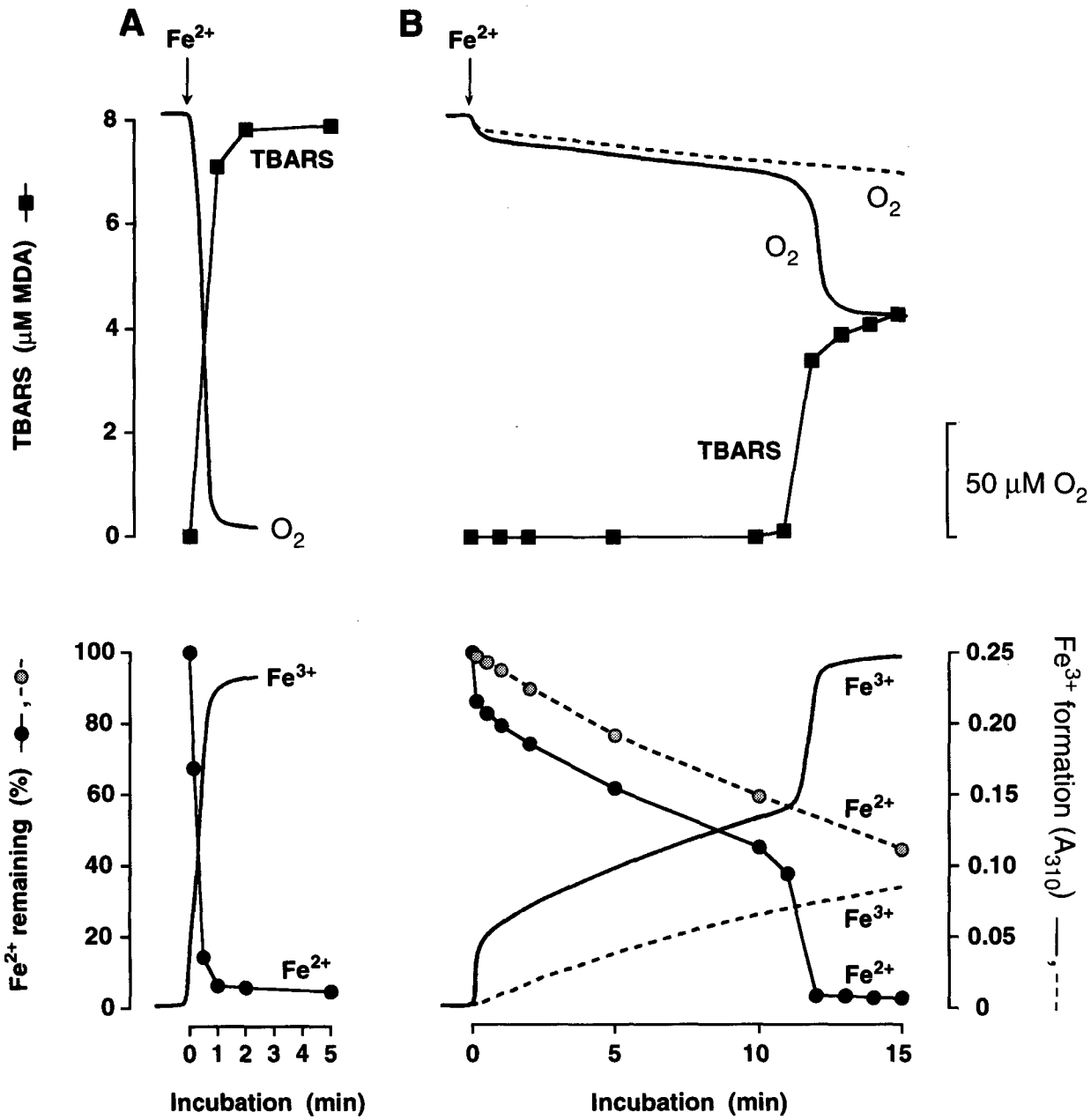


FIG. 4. Disappearance of Fe^{2+} during lipid peroxidation of PC(A) liposomes or PC(A)/PS liposomes. The incubation mixtures contained liposomes, 50 μM Fe^{2+}, 0.15 M KCl, and 25 mM Tris-HCl buffer at pH 7.5. The concentration of PC(A) liposomes, alone (A) and including 20 μM PS (B), were 0.1 mM. Oxygen consumption, TBARS production, Fe^{2+} concentration, and Fe^{3+} formation were determined as described in the Materials and Methods section, and the dotted lines indicate autoxidation of Fe^{2+} without phospholipid liposomes. The addition of Fe^{2+} is indicated by an arrow. These data were one of two separate sets of experiments which showed essentially similar results. TBARS, thiobarbituric acid-reactive substances. See Figure 1 for other abbreviations.

in the absence of oxygen was markedly lower than in the presence of oxygen. This implies that the possibility of direct reaction of Fe^{2+} with AAPH may be ruled out. Under air, no oxygen consumption, compared with that of AAPH alone was influenced by Fe^{2+} at various concentrations (data not shown), suggesting that A• may react faster with oxygen than with Fe^{2+}. These results suggest that Fe^{2+} is capable of scavenging peroxyl radicals. It is likely that the low rate of Fe^{2+} disappearance in the absence of oxygen reflects possible scavenging of A• and latent reaction of A• dimerization.

DISCUSSION

All differently charged amphipathic compounds chosen to determine the effect of charges in this study had saturated acyl chains. The ability of these compounds to lengthen lag peri-

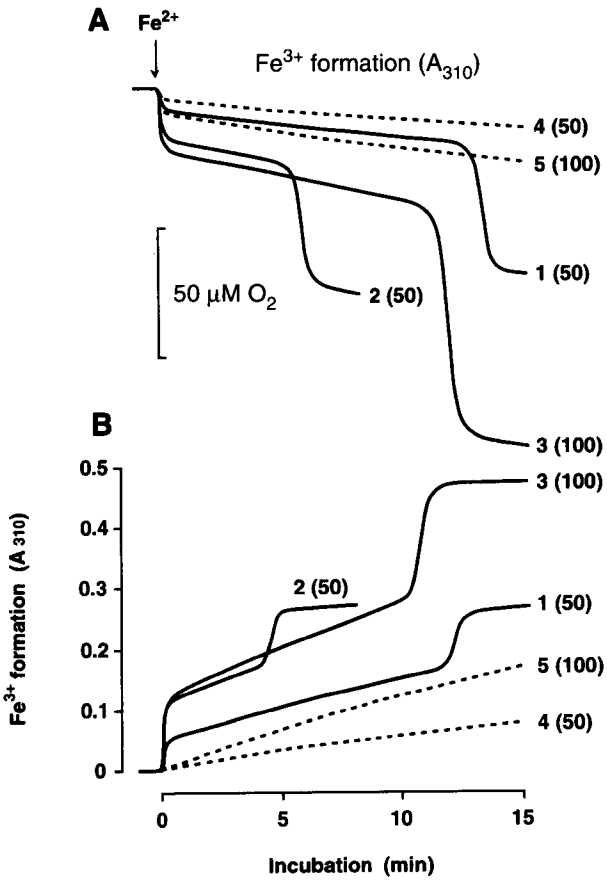


FIG. 5. Effect of lipid hydroperoxides on oxygen consumption (A) and Fe^{3+} formation (B) in Fe^{2+}-supported system containing PC(A)/PS liposomes. Liposomes, including preformed hydroperoxides, were prepared as described in the Materials and Methods section. The incubation mixtures contained 0.1 mM PC(A)/20 μM PS liposomes, including different amounts of hydroperoxides, 50 or 100 μM Fe^{2+}, 0.15 M KCl, and 25 mM Tris-HCl buffer at pH 7.5. The concentrations of hydroperoxides and Fe^{2+} added were 1, 2.6 μM and 50 μM; 2, 5.4 μM and 50 μM; 3, 5.4 μM and 100 μM, respectively. Dotted lines 4 and 5 indicate the autoxidation of Fe^{2+} at 50 μM and 100 μM in the absence of liposomes, respectively. The addition of Fe^{2+} is indicated by an arrow. This set of oxygraphs was one of three separate sets of experiments which showed essentially the same pattern. See Figure 1 for abbreviations.

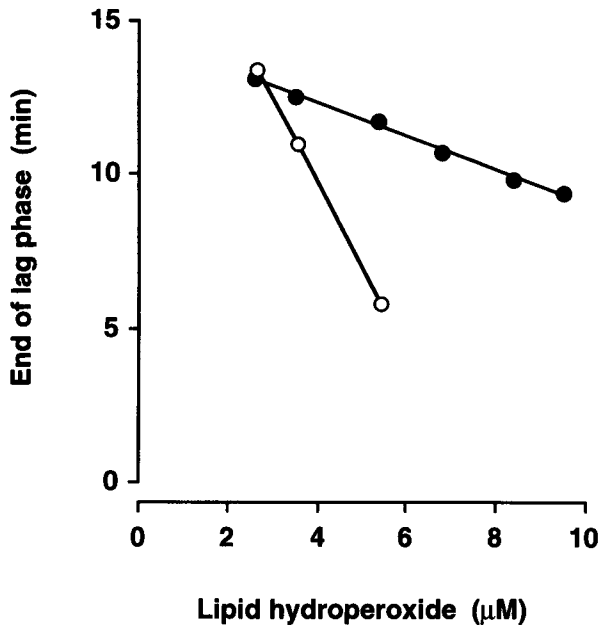


FIG. 6. Effect of lipid hydroperoxides on duration of the lag time in Fe^{2+}-supported system containing PC(A)/PS liposomes. Liposomes, including preformed hydroperoxides, were prepared as described in the Materials and Methods section. The composition of incubation mixtures is the same as in Figure 5, except that the concentrations of hydroperoxides included were 2.6, 3.5, 5.4, 6.8, 8.4, and 9.5 μM. ○, 50 μM Fe^{2+}; ●, 100 μM Fe^{2+}. Data represent the means of triplicate determinations. See Figure 1 for abbreviations.

ods in Fe^{2+}-supported oxygen consumption of PC(A) was in the order of PS>DCP>PC(DP). The effect of PS was predominantly superior to DCP, although the net-charge of these compounds is identical. One might imagine that the membrane instability of liposomes allows Fe^{2+} greater access to the internal lipid hydroperoxides resulting in differences in the lag phase. However, there were no differences in dye-leakiness among different liposomes used. Tadolini *et al.* (24) reported that phospholipid polar heads influence lipid hydroperoxide-dependent initiation of lipid peroxidation. Yoshida *et al.* (12) reported that saturated and unsaturated phosphatidylserine were similarly resistant to ferrous/ascorbate-supported lipid peroxidation, and they suggested the importance of their polar head groups in binding iron. Vile and Winterbourn (25) showed that iron also binds to the membrane of phosphatidylcholine liposomes. In our experiments, incorporation of 10–30 μM of PS, DCP, and PC(DP) into 100 μM PC(A) liposomes markedly produced lag periods, depending on their concentrations, regardless of the small amounts added. In addition, we postulated that the membrane surfaces might be saturated with Fe^{2+} at higher concentrations (Fig. 3). Hence, it is not plausible that prolongation of the lag period may largely depend on incorporation of saturated acyl chains into PC(A) liposomes. Our results appear to suggest that an increase in the anions on the liposomal surfaces affects the duration of the lag phase, supporting the view that liposomes with a net-negative surface charge are resistant to lipid peroxidation (11,12). However, the affinity of Fe^{2+} for the anions would be somewhat interfered with by the positive charges on the membrane surfaces. Indeed, the effect of PC(DP) on prolonging the lag period was poorer than that of DCP. In addition, the lag period (5 min) observed by adding a higher concentration (150 μM) of Fe^{2+} to PC(A) liposomes was significantly diminished by incorporating stearylamine (20 μM) with a positive charge into the liposomes (data not shown).

The total oxygen consumption after the lag phase clearly depended on the concentration of added Fe^{2+}, but inversely on PS concentrations. These observations suggest that the longer lag period leading to a decreased concentration of Fe^{2+}

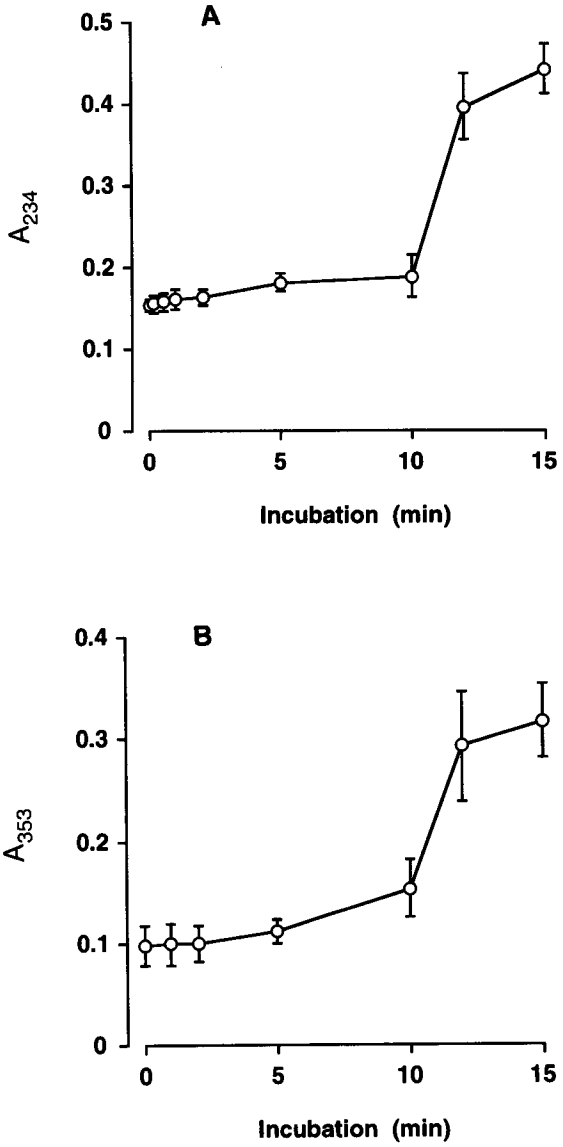


FIG. 7. Time-dependent changes of diene conjugation absorption (A) or hydroperoxide level (B) in PC(A)/PS liposomes with preformed hydroperoxides. Liposomes, including preformed hydroperoxides, were prepared as described in the Materials and Methods section. The incubation mixtures contained 0.1 mM PC(A)/20 μM PS liposomes including 5.7 μM hydroperoxides, 100 μM Fe^{2+}, 0.15 M KCl, and 25 mM Tris-HCl buffer at pH 7.5. Diene conjugation absorption was monitored spectrophotometrically by recording separately the increase in absorbance at 234 and 310 nm at 37°C, because Fe^{3+} formation gave an additional increase in absorbance at 234 nm. After measuring the spectral change at 234 and 310 nm in Fe^{2+} autoxidation, the contribution of absorbance at 234 nm due to Fe^{3+} formation was subtracted. The absorbance (A_{234}) of phospholipid liposomes free from hydroperoxides was extrapolated to be 0.020 from calibration of phospholipid liposomes including different concentrations of hydroperoxides. Hydroperoxide was determined by the method of Buege and Aust (15), after the reaction was stopped with 0.57 mM bathophenanthroline. Data represent the means ± SD of triplicate determinations. See Figure 1 for abbreviations.

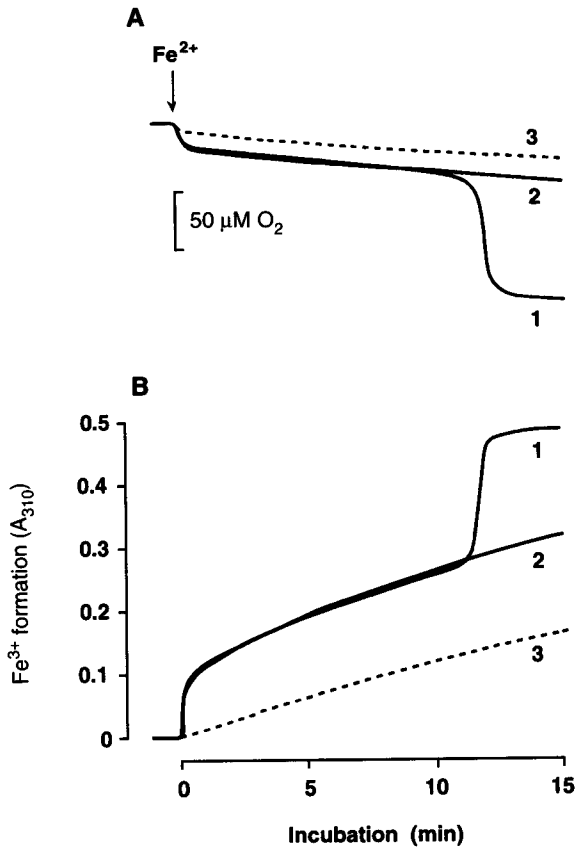
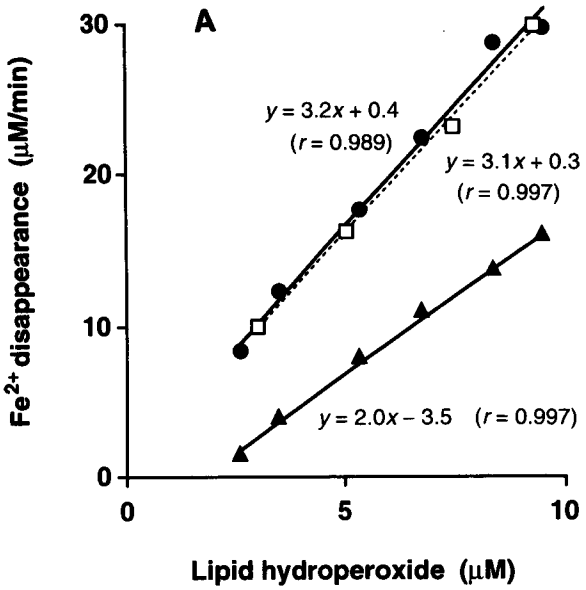


FIG. 8. Effect of *tert*-butylhydroxytoluene (BHT) in PC(A)/PS liposomes on Fe^{2+}-supported oxygen consumption (A) and Fe^{3+} formation (B). Liposomes, including preformed hydroperoxides, with or without BHT were prepared as described in the Materials and Methods section. The incubation mixtures contained 0.1 mM PC(A)/20 μM PS liposomes including 5.9 μM hydroperoxides, with or without 10 μM BHT, 100 μM Fe^{2+}, 0.15 M KCl, and 25 mM Tris-HCl buffer at pH 7.5. 1, without BHT; 2, with BHT; 3, without liposomes (Fe^{2+} autoxidation). The addition of Fe^{2+} is indicated by an arrow. These tracings were selected from four separate sets of experiments which showed essentially the same pattern. See Figure 1 for other abbreviations.

due to autoxidation results in diminishing the magnitude of the chain propagation. Thus, massive lipid peroxidation after a lag phase appears to occur at a critical concentration of Fe^{2+} on the membrane surface, as suggested by Driomina *et al.* (9). Minimum concentrations of Fe^{2+} extrapolated from lag periods in PC(A) liposomes, including different concentrations of PS (Fig. 3), further suggest that the critical concentration of Fe^{2+} might be substantially altered by the binding capacity of the membrane surfaces.

Samokyszyn *et al.* (1) observed that ADP-Fe^{2+}-dependent lipid peroxidation is preceded by a lag phase diminished or eliminated by adding increasing concentrations of ADP-Fe^{3+}. They suggested that Fe^{3+} is important to initiate lipid peroxidation at a 1:1 ratio of Fe^{2+} and Fe^{3+}, and the lag phase represents the time to obtain sufficient levels of ADP-Fe^{3+} from ADP-Fe^{2+} autoxidation. In our experiments, about half of the added Fe^{2+} was lost by the end of the lag time (Fig. 4). However, apart from ferric iron, other metals without a redox capacity can also stimulate lipid peroxidation (8,9), and Aruoma *et al.* (8) suggested that Fe^{3+} may interfere with the inhibitory reactions of Fe^{2+} by displacing it from certain sites on membranes.



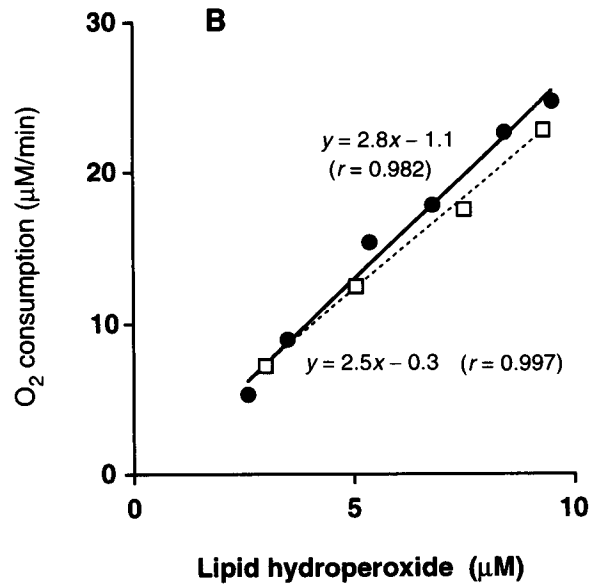


FIG. 9. Fe^{2+} disappearance (A) and oxygen consumption (B) in Fe^{2+}-supported system consisting of PC(A)/PS liposomes with different contents of lipid hydroperoxides. Liposomes, including preformed hydroperoxides, with or without BHT were prepared as described in the Materials and Methods section. The incubation mixtures contained 0.1 mM PC(A)/20 μM PS liposomes including different amounts of hydroperoxides, with or without 10 μM BHT, 100 μM Fe^{2+}, 0.15 M KCl, and 25 mM Tris-HCl buffer at pH 7.5. The concentrations of hydroperoxides were 3.0, 5.1, 7.5, and 9.3 μM, when BHT was incorporated into the liposomes, but without BHT the concentrations were the same as in Figure 6. Fe^{2+} disappearance and oxygen consumption were determined 1 min after incubation. In aerobic conditions, each point indicates Fe^{2+} disappearance and oxygen consumption after subtracting the portion of Fe^{2+} autoxidation. ●, without BHT under air; □, with BHT under air; ▲, without BHT under argon. Data represent the means of triplicate determinations. See Figures 1 and 8 for abbreviations.

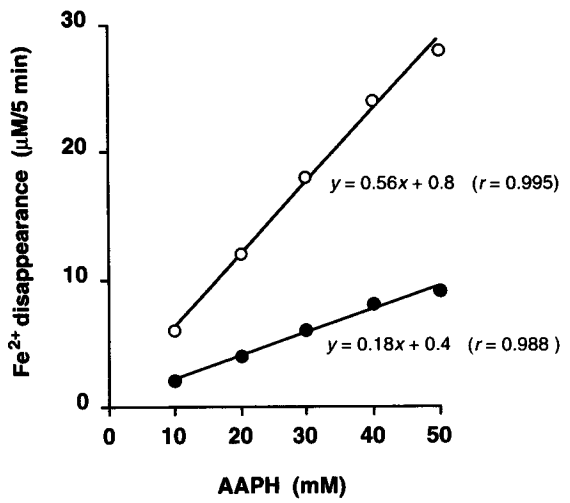


FIG. 10. Fe^{2+} disappearance promoted in the presence of 2,2′-azobis(2 amidinopropane)dihydrochloride (AAPH). The incubation mixtures contained AAPH at the indicated concentrations, 100 μM Fe^{2+}, 0.15 M KCl, and 25 mM Tris-HCl buffer at pH 7.5. The reaction was started by adding Fe^{2+} after preincubation for 1 min at 37°C. Under air, each point indicates Fe^{2+} disappearance after subtracting autoxidation of Fe^{2+}. ○, under air; ●, under argon. Data represent the means of triplicate determinations.

Driomina *et al.* (9) reported that an initial rise in malondialdehyde concentration accompanied by the fast flash of chemiluminescence occurred on adding Fe^{2+} to phospholipid liposomes. In this study, the initial disappearance of Fe^{2+} in Fe^{2+}-supported lipid peroxidation was also accompanied by oxygen consumption and was dependent on the lipid hydroperoxide content in the liposomes. Each molar ratio of either disappeared Fe^{2+} or consumed oxygen to preformed hydroperoxide in liposomes was constant, independent of the different amounts of lipid hydroperoxides. On the other hand, BHT incorporated into liposomes had no influence on the initial events of disappearance of Fe^{2+} and oxygen consumption. However, the results do not exclude the occurrence of free radical reactions on the membrane surface, because it is possible that the OH-group of BHT is located in membranes analogous to the location of α-tocopherol (26). Indeed, BHT completely inhibited the propagation of lipid peroxidation after the lag phase. These observations strongly support the results from the kinetics of Fe^{2+} oxidation in phosphatidylcholine liposomes, suggested by Garnier-Suillerot *et al.* (27), that hydroperoxide groups are localized at the aqueous interface and are decomposed by the subsequent binding of Fe^{2+} to the lipids. In Fe^{2+}-supported lipid peroxidation of PC(A)/PS liposomes with preformed hydroperoxides (10 μM), only 6% of 5(6)-carboxyfluorescein leaked by the end of the lag phase, while the propagation reaction was accompanied by the release of a great amount of dye (data not shown). These findings may suggest that a sequence of free radical reactions leading to the production of a lag phase preceding the propagation of lipid peroxidation in the lipid phase

occurs in the proximate region of the hydroperoxide group on the membrane surface of the liposomes.

Borg and Schaich (28,29) suggested that Fe^{2+} and Fe^{3+} at high concentrations can be expected to inhibit lipid peroxidation by the following radical scavenging reactions (Eqs. 1–3):

$$Fe^{2+} + LOO\bullet + H^+ \text{—fast} \rightarrow Fe^{3+} + LOOH \quad [1]$$

$$Fe^{2+} + LO\bullet + H^+ \text{—very fast} \rightarrow Fe^{3+} + LOH \quad [2]$$

$$Fe^{3+} + L\bullet \text{—fast} \rightarrow Fe^{2+} + L^+ \quad [3]$$

Equation 3 would be least important because of the short life time of L• except under severely hypoxic conditions (29). Our results from Fe^{2+} consumption in a model system, including AAPH, showed the possible involvement of Fe^{2+} in scavenging peroxyl radicals. However, whether Fe^{2+} has the ability to scavenge phospholipid peroxyl radicals remains unclear, as the redox potential of peroxyl radicals varies over a wide range (30).

The levels of diene conjugation and lipid hydroperoxides in Fe^{2+}-supported lipid peroxidation, did not change initially, regardless of a rise in oxygen consumption (Fig. 7). The lipid hydroperoxides used in this study were prepared by photooxidation. The oxidation of PC(A) with singlet oxygen produces eight hydroperoxide positional isomers, six (about 80%) of them having a conjugated diene (31). These conjugated dienyl hydroperoxides decompose through a series of reactions mediated by two moles of Fe^{2+} to form epoxy ketone and epoxy alcohol as major products (32,33). With regard to these reactions, Wilcox and Marnett (34) demonstrated that lipid alkoxyl radicals do not exist as reactive intermediates, since the intramolecular cyclization of lipid alkoxyl radicals into the adjacent site of unsaturation is instantaneous. Therefore, oxygen consumption, coupled with loss of conjugated dienes, results in epoxy peroxyl radical generation and subsequent formation of epoxy hydroperoxides (34). At the hydrogen abstraction stage, different types of compounds may compete. If epoxy peroxyl radicals abstract hydrogen from epoxy ketone and epoxy alcohol which still have a *bis*-allylic C-H bond in the molecule, the subsequent addition of oxygen generates new peroxyl radicals. During the sequence of these initial reactions in our study, three moles of Fe^{2+} and two moles of O_2 were consumed (Fig. 9). These results may be reconciled with assuming that Fe^{2+} scavenge the peroxyl radicals. When the above reactions are major reactions on the membranes, the levels of diene conjugation and hydroperoxide would not change significantly. The molar ratio of disappeared Fe^{2+} to preformed hydroperoxide contained in liposomes under argon was two. This ratio may be reasonable if Fe^{2+}, which could react with A• under argon, has the ability to scavenge epoxyallylic radicals that are more stable than alkoxyl radicals (34). Alternatively, both alkane radicals resulting from β-scission of alkoxyl radicals and lipid radicals generated by hydrogen abstraction from nonconjugated dienyl hydroperoxides might occur as minor (iron-independent) reactions at the oxygenation stage. The former reaction results in the loss of conjugated dienes, but the latter reaction is accompanied by conjugated diene formation.

Under the liposomal conditions, each hydroperoxide group in preformed PC(A) hydroperoxides appears to be located on the membrane surfaces, with its adjacent site available for lipid peroxidation inside the membrane near the surfaces. The overall reaction of Fe^{2+}-supported lipid peroxidation may be considered as follows. After adding Fe^{2+}, the initial hydroperoxide decomposition catalyzed by Fe^{2+} leads to secondary peroxyl radical generation accompanied by oxygen consumption. Subsequent reactions of Fe^{2+} on the membrane surfaces show antioxidant behavior, which depends on competitive local concentration of Fe^{2+} and is largely stoichiometric. These reactions, including a lag phase, may be explained by assuming Equation 4.

$$Fe^{2+} + LOO\bullet + H^+ \rightleftarrows Fe^{3+} + LOOH \quad [4]$$

The assumption includes that the forward reaction is fast, while the reverse reaction is slow. This is reasonable from kinetic (29,35) and thermodynamic considerations (30). At high levels, Fe^{2+} can effectively quench chain-carrying radicals competitively (forward reaction in Eq. 4), by intercepting them before chain propagation. Within the reaction cage, there remains no Fe^{2+}, and therefore, the lag phase might represent a slow reaction of Fe^{3+} with LOOH (reverse reaction in Eq. 4). Thus, the equilibrium in Equation 4 would lie toward the right during the lag phase, eventually leading to steady-state (apparent inhibition) of lipid peroxidation. When the concentration of Fe^{2+} falls, the Fe^{2+} lose their antioxidant ability and convert from antioxidant to prooxidant behavior as a result of redox cycling at trace levels of Fe^{2+}. In this context, a slow initiation phase in the presence of low peroxide concentration in heme-catalyzed lipid peroxidation is followed by a fast propagative phase once a threshold concentration of hydroperoxide is achieved (13,36). This would be the case for our experimental conditions. Peroxyl radicals on membranes penetrate into the lipid phase, and the lengths of the chain grow, resulting in massive lipid peroxidation.

Manganese ions are radical scavengers with a potent antioxidant ability (22). A comparative study of Mn^{2+} with Fe^{2+} may provide further information on the antioxidant processes of these metals.

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Oxysterols Found in Opacified Cornea of Fish

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ABSTRACT: We describe a new concept implicating oxidized cholesterol derivatives and very long-chain fatty acids as possible factors in the development of corneal opacification after death. Corneal tissues, removed from both fresh and stale fish eyes, were examined for cholesterol derivatives and fatty acids after methanolysis of lipids. Cholesta-3,5-dien-7-one, cholest-4-en-3-one, hexacosanoic acid, and hexacosenoic acid were identified *via* gas chromatography/mass spectrometry in opacified corneas, but not in significant amounts in fresh ones. The present study confirmed the presence of lipid hydrolysis and a peroxidation process in the opacified cornea.
Lipids 31, 1039–1043 (1996).

It is a widely held belief that fish is fresh so long as the cornea of the fish is clear. Corneal opacification is used in forensic science to indicate the time of death. It appears about 10 to 12 h after death (1). Although the cause of this phenomenon has not been elucidated, it has long been considered to be the result of drying of the cornea and protein degeneration.

Ocular tissues are continuously exposed to light, especially ultraviolet light, and are very susceptible to reactive oxygen species and free radicals. There is increasing interest in the idea that reactive oxygen species and free radicals have important roles in cataract development (2) and retinal degeneration (3) and, related to this, opacification of the lens has been found to be partly due to membrane damage of the cell by lipid peroxidation. These results suggest that lipid peroxidation is actively involved in corneal opacification as a result of membrane damage. To prove this hypothesis we used fish cornea, because of their easy availability.

The present study examined the peroxidation of cholesterol from fish cornea by means of gas chromatography/mass spectrometry (GC/MS).

MATERIALS AND METHODS

Hentriacontanoic acid methyl ester (31:0), used as an internal standard, 2-hydroxyhexacosanoic acid (hydroxy 26:0) and hexacosanoic acid methyl ester (26:0) were purchased from Sigma (St. Louis, MO), hydrogen chloride (5%, wt/vol) in methanol was obtained from Nachalai Tesque, Inc. (Kyoto, Japan), cholesta-3,5-dien-7-one, cholest-5-ene-3β, 7α-diol, cholest-5-ene-3β, 7β-diol, 3β-hydroxycholest-5-en-7-one, and cholest-4-en-3-one were purchased from Steraloids, Inc. (Wilton, NH). *cis*-17-Hexacosenoic acid methyl ester (26:1) was synthesized as follows: According to the conventional synthetic method, cyclooctanone was first subjected to Bayer-Villiger oxidation to give 8-hydroxyoctanoic acid methyl ester, which was then tosylated. Coupling reaction of oleyl magnesium bromide, with the prepared tosylate in the presence of Li_2CuCl_4, gave the desired 26:1 as colorless oil. The structure, including geometry of *cis*-stereochemistry, was firmly determined from H-nuclear magnetic resonance (300 MHz) spectrum, which exhibited two signals due to *cis*-olefinic protons (δ 5.34 and 5.35, dt, J = 10 and 5 Hz) with the coupling constant of $J_{17,18}$ = 10 Hz. All other chemicals were of analytical grade.

Fresh and clear corneas were removed just after death from several sea bream that had been brought live to a fish market; these were used as controls. Opacified corneas were obtained from several additional sea bream which had been stored for two days in the refrigerator, but were still suitable for consumption. Corneal tissue was minced with scissors, homogenized on ice with chloroform/methanol (2:1, vol/wt) solution, ultrasonically disintegrated, and extracted followed by centrifugation at 3,000 rpm for 15 min. Chloroform was evaporated under a nitrogen stream. The crude membrane lipids were placed in a screwcock-sealed reactivial with hydrogen chloride in methanol and heated at 120°C for 1 h to convert fatty acids to their methyl esters. Methanol was evaporated under a nitrogen stream and chloroform was added. An aliquot of 1 μL chloroform solution was injected into the GC/MS.

In vitro *experiment.* A sample of approximately 0.5 mg of each of cholest-5-ene-3β, 7α-diol, cholest-5-ene-3β, 7β-diol, and 3β-hydroxycholest-5-en-7-one was heated in a screwcock-sealed reactivial with 1 mL of hydrogen chloride in methanol for 1 h at 120°C. Excess reagents were evaporated to dryness under nitrogen stream. The residue was then dissolved in 250 μL of chloroform. A 1-μL aliquot of the sample was injected into the GC/MS apparatus.

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Abbreviations: EI, electron impact; GC/MS, gas chromatography/mass spectrometry; Rt, retention time; SIM, selected-ion-monitoring.

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GC/MS. GC/MS was performed on a Shimadzu Qp-1000 (Shimadzu, Kyoto, Japan) and a JMS DX-300 GC-MS (JEOL, Tokyo, Japan) connected on-line with a DA 5000 mass data analyzer (JEOL) fitted with a DB-1 fused-silica megabore column (30 m × 0.53 mm i.d., film thickness 1.5 μm; J&W Scientific, Folsom, CA). Helium was used as the carrier gas. The flow rate was 30 mL/min. The mass scan range was 50–600 atomic mass units, with a scan time of 3 s. The gas chromatograph oven temperature was programmed to 220–310°C at 4°C/min. The injection temperature was 240 °C; separation and insertion temperature, 240°C; ion-source temperature, 240 °C ; ionization voltage, 70 eV; ionization current, 60 μA; and acceleration energy, 3.0 kV.

Selected-ion monitoring (SIM) analyses were performed. The following ions were monitored: *m/z* 382, M^+ ion of cholesta-3,5-dien-7-one; *m/z* 384, M^+ ion of cholest-4-en-3-one; *m/z* 376, fragment ion of 26:1; *m/z* 410, M^+ ion of 26:0; and *m/z* 480, M^+ ion of internal standard. Standard curves were prepared by SIM analyses of 50 and 100 μg/mL of cholesta-3,5-dien-7-one and cholest-4-en-3-one with 50 μg/mL internal standard as well as 20, 40, and 80 μg/mL of 26:0 and 26:1 with 50 μg/mL internal standard. Signal areas of ions monitored were measured from the selected ion current trace and ratios of signal areas for *m/z* 382/480, 384/480, 376/480, and 410/480 were computed for standards and for lipid extracts of specimens.

RESULTS

Figure 1 shows total ion chromatograms performed for both opacified and control corneas. Cholesterol, cholesta-3,5-diene, and cholesteryl methyl ether were present in both samples. Many signals appeared in the opacified cornea. Electron impact (EI) mass spectra of signals A, B, C, and D are shown in Figure 2.

The signals A and B, appearing at a retention time (Rt) of 13.2 and 13.9 min, had a molecular ion at *m/z* 366 and a fragment ion at *m/z* 211. After methanolysis of standard cholest-5-ene-3β, 7α-diol and cholest-5-ene-3β, 7β-diol *in vitro,* we observed three cholestatrienes (cholesta-2,4,6-triene, cholesta-3,5,7-triene, and unknown) with a molecular ion at *m/z* 366 and a fragment ion at *m/z* 211. Two signals in the analysis of each of three cholestatrienes were consistent with signals A and B from the opacified cornea. Electron impact mass spectra of standard cholesta-3,5-dien-7-one and cholest-4-en-3-one are also shown in Figure 2. After methanolysis of standard 3β-hydroxycholest-5-en-7-one, we observed one signal being consistent with standard cholesta-3,5-dien-7-one. The mass spectrum of signal C in Figure 2 (Rt, 16.6 min) was consistent with that of standard cholesta-3,5-dien-7-one (*m/z* 382, 174), and the mass spectrum of signal D (Rt, 17.1 min) was consistent with that of standard cholest-4-en-3-one (*m/z* 384, 124).

Mass chromatograms that were monitored at *m/z* 366, 382, and 384 are shown in Figure 3. Thus, signal C was identified as cholesta-3,5-dien-7-one and signal D as cholest-4-

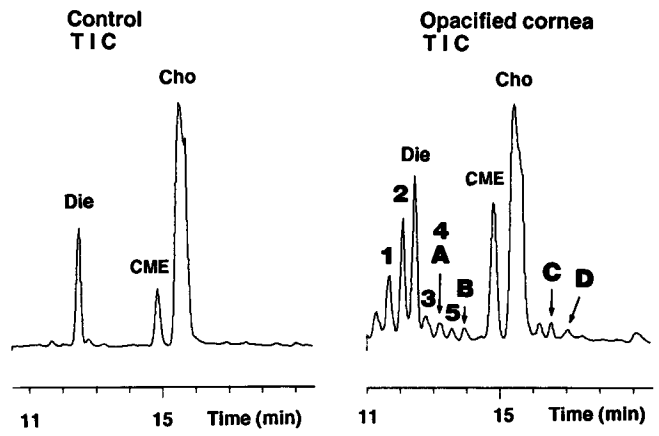


FIG. 1. Total ion chromatograms (TIC) for control and opacified cornea. Die, cholesta-3,5-diene; CME, cholesteryl methyl ether; Cho, cholesterol. A, B, unidentified but may be cholesta-2,4,6-triene and cholesta-3,5,7-triene; C, cholesta-3,5-dien-7-one; D, cholest-4-en-3-one; 1, 2-hydroxytetracosenoic acid (h24:1); 2, 2-hydroxytetracosanoic acid (h24:0); 3, hexacosenoic acid (26:1); 4, hexacosanoic acid (26:0); and 5, 2-hydroxypentacosanoic acid (h25:0).

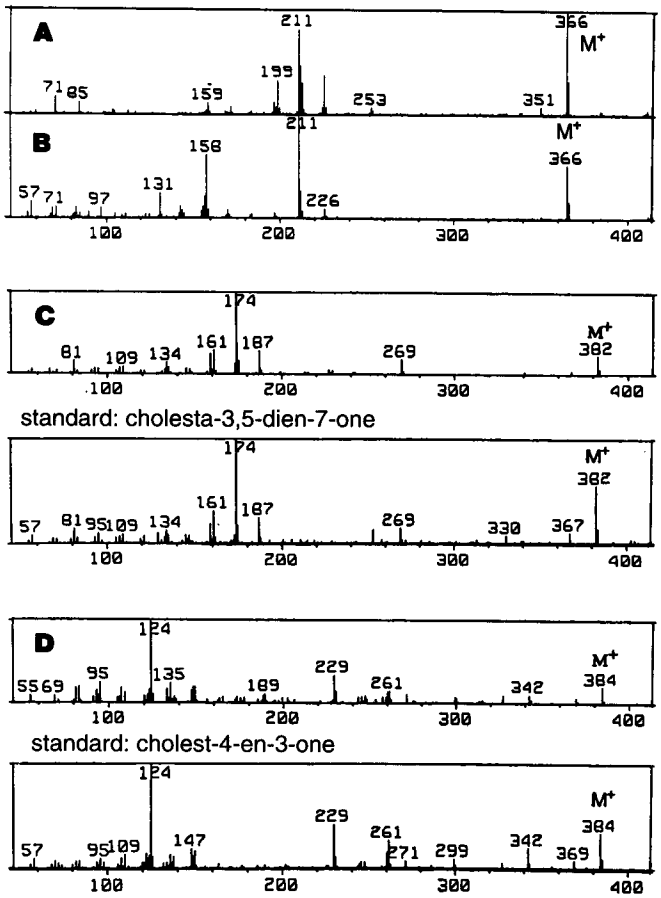


FIG. 2. Electron impact mass spectra of standard cholesta-3,5-dien-7-one and cholest-4-en-3-one, and for signals A, B, C, and D.

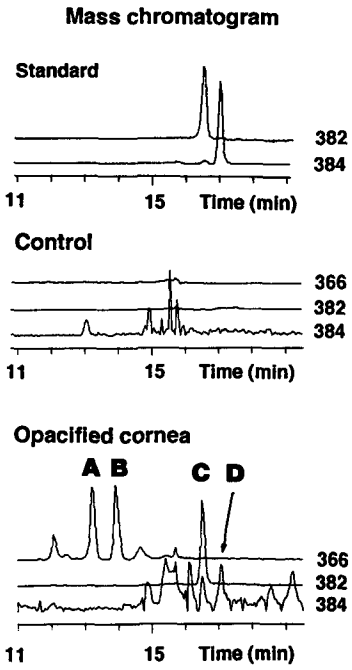


FIG. 3. Mass chromatograms with monitoring at *m/z* 366, 382, and 384 for standard cholesta-3,5-dien-7-one and cholest-4-en-3-one, control cornea and opacified cornea.

en-3-one by both mass spectra and mass chromatograms. Many unidentified signals appeared by monitoring at *m/z* 384 for the opacified cornea samples in Figure 3. Of these, the left signal of C (Rt, 16.1 min) might be cholest-5-en-3-one, but we failed to get a clear mass spectrum of it. The levels of cholesta-3,5-dien-7-one and cholest-4-en-3-one were 4.8 ± 0.2 μg/mg fat and 4.5 ± 0.1 μg/mg fat in the opacified cornea (n = 5); they were not detected in significant amounts in the control cornea.

Next, EI mass spectra of signals 1, 2, 3, 4, and 5 in Figure 1 are shown in Figures 4 and 5. The mass spectrum associated with signal 1 was consistent with 2-hydroxytetracosenoic acid methyl ester (*m/z* 337, 396), signal 2 was consistent with 2-hydroxytetracosanoic acid methyl esters (*m/z* 339, 398), and signal 5 was 2-hydroxypentacosanoic acid methyl ester (*m/z* 353, 412). The signals originating from

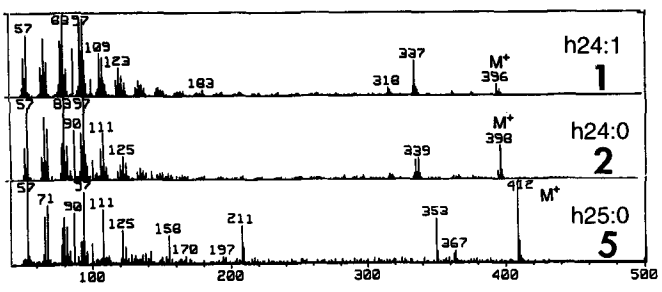


FIG. 4. Electron impact mass spectra of h24:1 for signal 1, h24:0 for signal 2, and h25:0 for signal 5 in Figure 1.

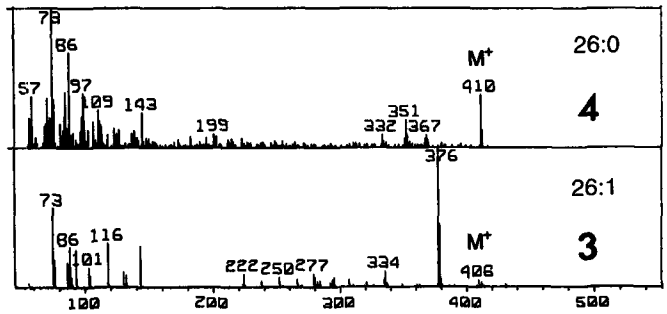


FIG. 5. Electron impact mass spectra of 26:0 for signal 4 and 26:1 for signal 3 in Figure 1.

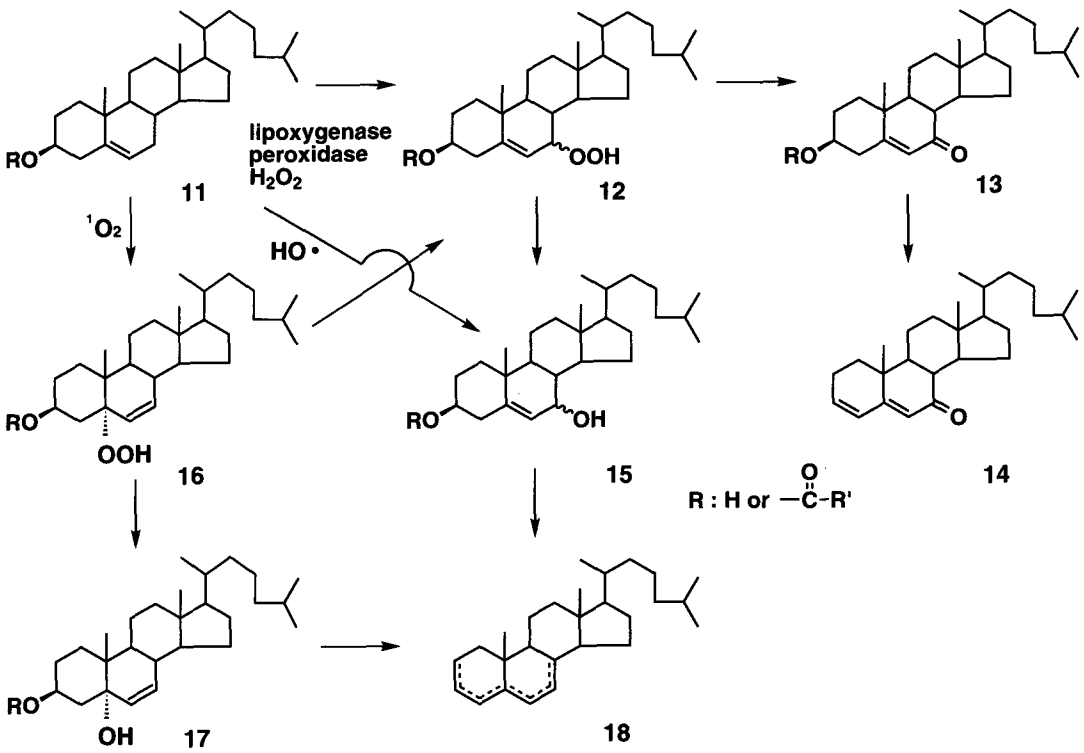
methyl esters of standard *cis*-17-26:1 and 26:0 were seen at Rt of 12.8 and 13.1 min, respectively. The mass spectrum of signal 3 (Rt, 12.8 min) was consistent with *cis*-17-26:1 (*m/z* 408, 376), and signal 4 (Rt, 13.1 min) was consistent with 26:0 (*m/z* 410, 367), as shown in Figure 5. The levels of 26:0 and 26:1 were 3.0 ± 0.1 and 6.2 ± 0.2 μg/mg fat in the opacified cornea (n = 5); they were not detected in significant amounts in the control cornea.

DISCUSSION

The results of the present study show for the first time that the opacified cornea of fish contained significant amounts of cholesta-3,5-dien-7-one, cholest-4-en-3-one, and cholestatrienes, whereas these compounds were essentially absent from the normal cornea of fish. The structures were confirmed by their mass spectra on GC/MS.

Many authors have written biological effects of oxysterols such as cytotoxicity, atherogenicity, sterol metabolism interference, mutagenicity, and carcinogenicity (4–7). Analyses of lipids of erythrocyte membrane (8) and fatty liver (9) of alcoholic patients revealed a significant accumulation of cholesta-3,5,-dien-7-one. In addition, elevated cholester-5-ene-3β, 7β-diol was seen in human atheroma (10) and 7-hydroperoxycholest-5-en-3β-ol was recognized in oxidized human low density lipoprotein (11).

An outline of oxidized cholesterol formation is shown in Scheme 1. The first step may be a peroxidation of cholesterol [11] to 7-hydroperoxycholesterol [12] in a microsomal lipid oxidizing system or by lipoxygenase and peroxidase, or cholesterol [11] to 5α-hydroperoxycholest-6-en-3β-ol [16] by singlet oxygen followed by rearrangement to 7-hydroperoxycholesterol [12]. This [12] might be oxidized to 3β-hydroxycholest-5-en-7-one [13], followed by dehydration to cholesta-3,5-dien-7-one [14], or might be reduced to cholest-5-ene-3β,7α-diol [15] and cholest-5-ene-3β,7β-diol [15]. These [15] might be dehydrated to cholestatrienes [18] during the acid methanol treatment. In contrast, [16] might be reduced to cholest-6-ene-3β, 5α-diol [17], followed by dehydration to cholestatriene [18]. Hydroxyl radical generated by Fenton reaction may form cholest-5-ene-3β, 7α and 7β-diol [15]. Cholest-5-ene-3β, 7β-diol and 3β-hydroxycholest-



SCHEME 1

5-en-7-one appeared at Rt of 18 and 19.7 min, respectively; however, we failed to detect these signals in Figures 1 and 3 because of acid methanol treatment.

In contrast, Cenedella *et al.* (12) detected cholesta-3,5-diene in an opacified dog cornea without the use of saponification. Detecting more than five peaks by GC/MS after heating cholesta-3,5-dien-7-one at 70°C for 30 min, we adopted methanolysis. However, a recent kinetic study on 3β-hydroxycholest-5-en-7-one stability during saponification clearly showed that 3β-hydroxycholest-5-en-7-one destruction occurred rapidly at 65 and 75°C (13). We should have used "cold" saponification at 45°C as a preliminary experiment to avod formation of artifacts. Because we detected cholesta-3,5-diene, as well as cholesteryl methyl ether, even in the control specimen under methanolysis conditions, and these steroids may not have been present in the initial extract before methanolysis, it is not clear whether cholesta-3,5-diene is present in the opacified cornea or not.

Next, we considered the incidence of lipid peroxidation in the cornea after death. In general, superoxide dismutase and reduced glutathione must act as agents of a radical scavenging system, whereas catalase scavenges hydrogen peroxide. Aldehyde dehydrogenase in mammalian lens has been reported to have a role in preventing cataract formation (14). Moreover, corneal alcohol dehydrogenase and aldehyde dehydrogenase isozymes were also identified as having a role in the detoxification of lipid peroxidation (15). When the lens was incubated *in vitro,* using xanthine oxidase as a superoxide generating system, cataract formation was suppressed by adding vitamin E (16). In contrast, reduced glutathione concentration, as well as reduced glutathione activity in the lens, has been shown to cause senile cataracts (17,18). We suggest that the activity of various radical scavenging systems decreases rapidly after death and, consequently, lipid peroxidation cannot be prevented, contributing to corneal opacification.

In the present study, an overlap of signal A, the elimination reaction product, with methyl ester of fatty acid (26:0), unfortunately was observed as the other instance of overlap of cholesta-3,5-diene with fish fatty acids (19). Although this study revealed the elevation of not only very long chain fatty acids but 2-hydroxy fatty acids, the implication of 2-hydroxy fatty acid generation on corneal opacification was not be elucidated clearly, however, hydrolysis of esterified fatty acids to their free form occurs under these post-mortem conditions.

As very long-chain fatty acids are degraded by the peroxisomal β-oxidation system, peroxisomal disorders such as Zellweger syndrome have been reported to cause the accumulation of these acids in plasma or tissue (20). Thus, the accumulation of 26:0 and 26:1 in the opacified cornea suggests the occurrence of peroxisomal disorders at the early stage of death.

The signal originating from methyl ester of 2-hydroxyhexacosanoic acid (hydroxy 26:0) (*m/z* 426, 367) appeared at Rt of 15.2 min; however, we failed to get this signal in Figure 1 because of an overlap with cholesterol so that it is not clear whether the opacified cornea had hydroxy 26:0.

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Involvement of Phospholipid Molecular Species in Controlling Structural Order of Vertebrate Brain Synaptic Membranes During Thermal Evolution

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ABSTRACT: Fluorescence anisotropy parameter of [p-(6-phenyl)-1,3,5-hexatrienyl]phenyl–propionic acid (DPH–PA) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA–DPH) embedded in synaptic plasma membranes prepared from brains of cold (5°C) and warm (22°C) adapted fish (*Cyprinus carpio* L.), rat *(Rattus norvegicus)* and bird *(Branta canadensis)*, was studied. Fatty acid composition of total lipids as well as molecular species composition of diacyl phosphatidylcholines and phosphatidylethanolamines was also determined. The amount of long-chain polyunsaturated fatty acids decreased with increasing body temperature. There was a near-complete compensation of membrane structural order for environmental/body temperature over the evolutionary scale as seen by DPH–PA. Using TMA–DPH, the compensation was partial with rat and bird. Since DPH–PA and TMA–DPH differ in their charges, it is proposed, that the former reported membrane regions rich in cationic or zwitterionic (neutral) phospholipids and the latter, membrane regions rich in negatively charged phospholipids in the synaptic plasma membranes. Many different molecular species (20–25) of diacyl phosphatidylcholines and diacyl phosphatidylethanolamines were identified. The level of 16:0/22:6 phosphatidylcholine decreased while disaturated phosphatidylcholines increased with increase of environmental/body temperature from the fish through the bird. Level of 1-monoenoic, 2-polyenoic phosphatidylethanolamines also decreased with an increase in environmental/body temperature. Experiments using vesicles made of mixed synthetic phosphatidylcholine vesicles (16:0/16:0, 16:0/18:1, 16:0/22:6 in various proportions) showed that increase in disaturated phosphatidylcholine species does not explain the observed complete adjustment of membrane structural order in synaptic plasma membranes. Change in level of 1-monoenoic, 2-polyenoic phosphatidylethanolamines might be one of the factors involved in controlling the biophysical properties of the membrane according to the temperature.
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Convincing evidence has been presented that vertebrates possess the ability to adjust the fluidity of their membranous structure to environmental/body temperature in order to maintain their structural and functional integrity (1). Biological membranes are assemblies of lipids and proteins (2), and the lipid component is regarded to be responsible for controlling the physical properties of these structures. Although the motional properties of the hydrocarbon chains of the component polar lipids define the actual "fluidity" of membranes, the precise role of the individual membrane lipid constituents is not completely understood. Accumulation of polyunsaturated fatty acids in structural lipids during adaptation to reduced temperatures has repeatedly been demonstrated (3–5), but they may not be entirely responsible for the increase of fluidity since the physical properties of phospholipids are not linear functions of the number of double bonds present (6,7). Indeed, a number of data show no increase in fluidity in membranes of intact cells (8–11) or subcellular membranes (12,13) rich in polyunsaturated fatty acids. Earlier we observed the accumulation of 1-monounsaturated, 2-polyunsaturated phosphatidylethanolamine molecular species in livers and brains of freshwater and marine fish (14,15) and shrimp (16) species evolutionary or seasonally adapted to their thermal environment and concluded that not the gross amounts of polyunsaturated fatty acids but their combination with monounsaturated fatty acids in certain structural lipids play a role in controlling membrane physical properties in response to changes of body/environmental temperature. The synaptic membrane fraction of vertebrate brains has been shown to exhibit an almost perfect compensation for the temperature over the evolutionary scale (17). It seemed plausible to compare the molecular species composition of the two major diacyl phosphoglycerides, phosphatidylcholines, and phosphatidylethanolamines, with its biophysical properties in representative

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Abbreviations: DPH–PA, 3-[p-(6-phenyl)-1,3,5-hexatrienyl]phenyl–propionic acid; TMA–DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene; POPC, 1-palmitoyl, 2-oleoyl phosphatidylcholine (16:0/18:1); DPPC, 1-palmitoyl, 2-palmitoyl phosphatidylcholine (16:0/16:0); PDPC, 1-palmitoyl, 2-docosahexaenoyl phosphatidylcholine (16:0/22:6); SPM, .

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species of fish, adapted to summer and winter temperatures, as well as of warm-blooded animals, such as mammalians and birds.

MATERIALS AND METHODS

Animals. Winter- (5°C) and summer adapted (22°C) carp (*Cyprinus carpio* L.) weighing 1–1.5 kg, collected from a local fish farm in the course of years 1994–1996; male Wistar rats (160 g); and wild geese *(Branta canadensis),* hunted near Pasco Washington, were used for preparation of brain synaptic membranes. Brains were removed immediately after killing the animals by decapitation or hunting and placed into liquid nitrogen until processed. Pooled brain probes of ten carps, five rats, and five birds were used for the investigations.

Synaptic plasma membrane fraction was prepared according to Szücs and Coscia (18). Briefly, the brains (6–7 g) were homogenized in 0.32 M sucrose containing 1 mM $MgCl_2$ 1 mM EDTA, 50 mM Tris HCl, and pH 7.1 using a Teflon homogenizer, ten passes. The homgenate was centrifuged at $1000 \times g$ for 10 min. The supernatant was sedimented at $13000 \times g$ for 30 min and resuspended in sucrose solution and spun again at $10800 \times g$ for 20 min followed by a further supension and centrifgation at $14000 \times g$ for 20 min. Then, the pellet was suspended by a Dounce homogenizer and lyzed at 0°C for 30 min. Finally the membranes were overlaid to a sucrose gradient consisted of a 10, 28.5, and 34% sucrose and centrifugated using a Beckman SW 27 rotor (Beckman Instruments, Fullerton, CA) for 60 min. The synaptic membranes were harvested at the interface of 28.5 and 34% sucrose and stored at −70°C. Electron microscopy showed formation of sealed vesicles free of mitochondrial contamination.

Analysis of lipids. Lipids were extracted from synaptic plasma membranes according to Folch *et al.* (19). Lipid extracts were further fractionated by thin-layer chromatography on precoated silica gel plates (Silica Gel G 60; E. Merck, Darmstadt, Germany) according to Fine and Sprecher (20). For preparation of choline and ethanolamine phosphoglycerides the spots were visualized by spraying with 0.5% 1-anilino,8-naphthalene sulphonic acid (Sigma, St. Louis, MO) in 50% methanol and viewed under ultraviolet light. They were then removed and extracted with chloroform/methanol 2:1, or used directly for derivatization to determine their molecular species compositions. Molecular species composition was determined according to Takamura *et al.* (21). Separation of purified dinitrobenzoyl derivatives of the diacylglycerols, obtained by phospholipase C (*Bacillus cereus*, Sigma) was performed on a Hypersyl column (5 μ, 250 × 4.6 mm) using a Merck-Hitachi 6220 high-performance liquid chromatography (HPLC) instrument.

Membrane fluidity. The synaptic plasma membranes or phosphatidylcholine vesicles were labeled either with 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA–DPH) or 3-[p-(6-phenyl)-1,3,5-hexatrienyl]phenyl–propionic acid (DPH–PA) or DPH–PA, respectively, 15 min prior to starting the measurements. Steady-state fluorescence anisotropy parameter, R_{ss}, was determined between 2 and 30°C at 2°C intervals or at the respective body temperatures of the animals using a computer-controlled Hitachi MPF 2A spectrophotofluorimeter in heating mode (1°C/min) (22). Steady-state fluorescence anisotropy parameter was calculated according to Equation 1:

$$R_{ss} = I_{(vv)} - Z \cdot I_{(vh)} + Z \cdot I_{(vh)} \quad [1]$$

where $I_{(v)}$ and $I_{(h)}$ are the vertically and horizontally polarized light intensities, respectively, while $Z = 0.92$ and was calculated according to the formula: $Z = I_{(vh)}/I_{(hh)} \cdot R_{ss}$ primarily reflects the degree of hindrance to free rotation of the probe in its environment (23). Higher values represent more ordered, less fluid structures. Each data point is the average of ten readings.

Chemicals. DPH–PA and TMA–DPH were from Molecular Probes, Inc. (Eugene, OR). 1-palmitoyl, 2-palmitoyl phosphatidylcholine (16:0/16:0) (DPPC), 1-palmitoyl, 2-oleoyl phosphatidylcholine (16:0/18:1) (POPC), and 1-palmitoyl, 2-docosahexaenoyl phosphatidylcholine (16:0/22:6) (PDPC) were from Avanti Polar Lipids, Inc. (Birmingham, AL). All reagents were of analytical or HPLC grade.

RESULTS

Table 1 lists the fatty acids present in total lipids of brain synaptic plasma membranes of warm- and cold-adapted fish, rat, and goose. There is a clear tendency of diminishing the level of 22:6, with increasing environmental/body temperature.

TABLE 1
Fatty Acid Composition of Total Lipids in Synaptic Plasma Membranes of Representative Vertebrate Species[a]

Fatty acid	CA-Fish	WA-Fish	Rat	Bird
12:0	2.02	7.91	1.40	6.52
14:0	1.69	9.94	1.64	9.48
14:1	2.26	4.44	0.79	0.10
15:0	0.30	2.52	0.74	0.14
15:1	0.17	0.62	—	—
16:0	20.59	20.05	20.30	23.39
16:1	1.94	2.42	0.51	1.14
16:2	0.14	1.65	0.22	1.76
16:3	0.26	1.38	2.69	—
18:0	11.64	10.64	19.81	20.90
18:1n-9	16.98	14.84	17.35	13.71
18:2n-6	6.01	2.57	2.31	0.46
18:3n-3	0.46	—	0.80	0.45
18:4n-3	1.76	0.60	—	—
20:2n-6	0.23	—	0.80	1.00
20:3n-3	—	0.54	0.55	0.58
20:4n-6	6.09	3.02	9.82	7.04
20:5n-3	0.66	1.38	—	—
22:4n-6	0.26	0.63	0.96	1.80
22:5n-6	0.94	0.54	3.27	0.24
22:5n-3	1.23	1.35	3.11	0.24
22:6n-3	24.14	12.90	12.61	10.69

[a]CA-Fish: cold-adapted fish, WA-Fish: warm-adapted fish.

TABLE 2
Molecular Species Composition of Diacyl Phosphatidylcholines in Synaptic Plasma Membranes of Representative Vertebrate Species[a]

Molecular species	CA-Fish	WA-Fish	Rat	Bird
22:6/22:6	1.14 ± 0.45	0.24 ± 0.10	—	—
22:6/20:5	0.18 ± 0.08	—	—	—
20:4/20:4	0.94 ± 0.37	0.84 ± 0.60	—	—
16:1/16:1	—	—	0.67	0.78
18:1/22:6	2.35 ± 0.72	1.98 ± 1.00	1.18	0.68
16:0/22:6	13.25 ± 2.81	9.87 ± 3.30	4.51	3.53
18:1/20:4	1.71 ± 0.42	0.68 ± 0.32	2.02	0.59
16:0/20:4	2.72 ± 0.54	2.68 ± 1.27	5.91	2.27
18:0/20:5	0.85 ± 0.24	0.35 ± 0.20	4.40	—
18:0/22:6	5.66 ± 0.88	4.54 ± 1.35	4.49	20.27
18:0/18:2	14.66 ± 0.97	16.47 ± 2.65	1.27	—
18:0/20:4	2.10 ± 1.36	1.00 ± 0.65	4.76	2.99
18:0/22:5	0.55 ± 0.20	2.01 ± 0.90	—	0.80
18:1/18:1	3.55 ± 0.53	3.28 ± 0.80	4.40	0.94
16:0/18:1	33.50 ± 1.94	39.20 ± 6.50	34.44	29.62
16:0/16:0	4.72 ± 2.51	8.71 ± 2.87	17.17±	29.57
18:0/18:1	1.04 ± 0.55	0.25 ± 0.20	9.27	5.67
16:0/18:0	8.05 ± 1.25	9.79 ± 3.65	4.20	4.29
18:0/18:0	2.09 ± 1.60	—	—	—

[a]WA: warm-adapted (n = 3, mean ± SD), CA: cold-adapted (n = 4, mean ± SD).

Tables 2 and 3 give the molecular species composition of diacyl-phosphatidylcholines and ethanolamines in the synaptic membranes. Species of 16:0/18:1 dominated the diacyl-phosphatidylcholine fraction in the cold-adapted fish followed by 16:0/22:6. 16:0/16:0, and 18:0/22:6 were represented by low values compared to animals with warmer body/environmental temperatures. An increasing tendency was observed in the level of 16:0/16:0 phosphatidylcholines from cold-adapted fish through bird (from 5 to 27%). Parallel to this, the level of 16:0/22:6 decreased from 14.5 to 3.5% in cold-adapted fish to rat (Table 2).

TABLE 3
Molecular Species Composition of Diacyl Phosphatidylethanolamines in Synaptic Plasma Membranes of Representative Vertebrate Species[a]

Molecular species	CA-Fish	WA-Fish	Rat	Bird
22:6/22:6	6.00 ± 1.06	3.21 ± 1.21	—	—
22:6/22:5	1.59 ± 0.60	1.39 ± 1.01	—	—
20:4/20:4	1.41 ± 0.50	1.28 ± 1.00	—	—
18:1/20:5	0.98 ± 0.58	—	—	0.92
18:1/22:6	13.45 ± 2.60	6.56 ± 3.73	2.44	2.59
16:0/22:6	14.94 ± 2.80	14.91 ± 1.73	8.76	9.96
18:1/20:4	6.89 ± 0.45	3.48 ± 1.08	3.07	1.12
16:0/20:4	4.65 ± 1.09	4.14 ± 1.08	3.08	1.89
18:0/20:5	2.34 ± 0.38	0.43 ± 0.20	1.42	0.55
18:0/22:6	20.48 ± 1.35	33.61 ± 1.06	32.86	47.67
16:0/18:2	3.19 ± 1.00	1.18 ± 0.70	0.91	—
18:0/22:5	0.25 ± 0.19	1.23 ± 0.60	5.40	1.67
18:0/20:4	9.50 ± 1.11	9.57 ± 1.51	23.37	20.06
18:1/18:1	4.70 ± 0.59	4.77 ± 1.77	3.09	0.37
16:0/18:1	4.11 ± 0.49	4.77 ± 1.72	4.20	3.54
16:0/16:0	1.23 ± 0.74	2.87 ± 1.39	4.69	5.84
18:0/18:1	0.82 ± 0.50	0.34 ± 0.15	4.79	4.33
16:0/18:0	1.22 ± 0.34	4.24 ± 2.97	0.91	0.41
18:0/18:0	0.34 ± 0.21	1.63 ± 0.48	—	—

[a]See Table 1 for abbreviations.

In diacyl-phosphatidylethanolamine fraction of cold-adapted fish 18:0/22:6, 18:1/22:6, and 16:0/22:6 were the major species. Increase in environmental/body temperature was accompanied by an increase in the level of 18:0/22:6 and a decrease in 18:1/22:6, 18:1/20:4, and 16:0/22:6 (Table 3). Proportion of 16:0/20:4 was almost identical in the different vertebrate classes while the level of 18:0/20:4 showed an abrupt increase with type of control of body temperature, that is the shift from poikilotherm to homeotherm (Table 3). Dipolyunsaturated (22:6/22:6, 20:4/20:4) molecular species were detected only in fish probes being the cold-adapted ones more rich in them than the warm-adapted fish.

Table 4 the steady-state anisotropy parameters (R_{ss}) of DPH–PA and TMA–DPH embedded in brain synaptic plasma membranes of the representative vertebrate classes is compared at their respective body temperatures. As seen, DPH–PA reported identical fluidities at the respective body temperatures, indicating a perfect compensation of membrane structural order for the environmental/body temperature. In contrast, TMA–DPH gave a different response and reported more fluid structures with rat and bird than expected on the basis of results obtained by DPH–PA.

Figure 1 shows the effects of different phosphatidylcholine species, showing the most characteristic changes during the thermal evolution, on structural order of fatty acids in phospholipid vesicles. Three vesicle populations were prepared from PDPC, POPC and DPPC, one representing the fish (PDPC/POPC/DPPC, 53:35:12%) the other one the rat (PDPC/POPC/DPPC, 35:45:20%), and the third one the bird (PDPC/POPC/DPPC, 28:37:35%) synaptic plasma membranes. Since phosphatidylcholine is one of the major constituents of these structures, it was expected that the results obtained will partially clarify the biological significance of the observed changes shown in Table 4. As seen, the "fish-like" phosphatidylcholine combination gave the least ordered, more fluid vesicles, and the "bird like" combination the most ordered, less fluid, structures at low measuring temperatures. At high measuring temperatures, this difference is reduced.

TABLE 4
Steady-State Fluorescence Anizotropy Parameter, R_{ss}, of 1-(4-Trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA–DPH) and 3-[p-(6-Phenyl)-1,3,5-hexatrienyl]phenyl–propionic acid (DPH–PA) Embedded in Synaptic Plasma Membranes of Representative Vertebrate Species Measured at Their Body Temperatures[a]

Species	T_{body} °C)	DPH–PA	TMA–DPH
Winter carp (n = 4)	5	0.263 ± 0.015	0.262 ± 0.010
Summur carp (n = 3)	23	0.280 ± 0.021	0.244 ± 0.019
Rat (n = 4)	37	0.265 ± 0.010	0.221 ± 0.010
Bird (n = 3)	41	0.263 ± 0.015	0.182 ± 0.020

[a]n = Number of probes tested. Values are the means of ten readings ± SD. on each data point.

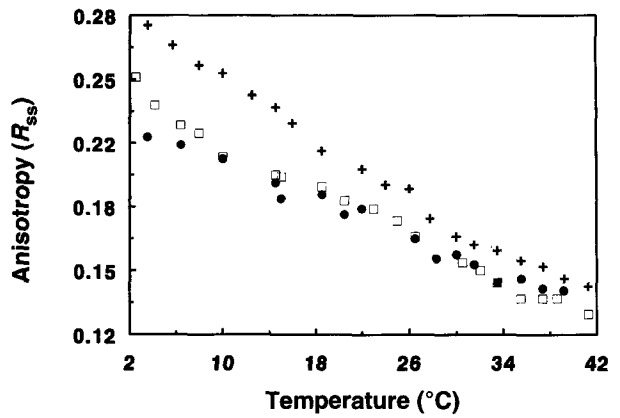


FIG. 1. Temperature dependence of fluorescence anizotropy parameter, R_{ss}, of DPH–PA embedded in "fish-like" (DPPC/POPC/PDPC, 12:35:53%); "rat-like" (DPPC/POPC/PDPC, 20:45:35%); and "bird-like" (DPPC/POPC/PDPC, 35:37:28%) phosphatidylcholines. ●: "fish-like," □: "rat-like," +: "bird-like" phosphatidylcholine vesicles. Each data point is the average of ten readings.

DISCUSSION

The experiments presented in Tables 1 and 4 confirm the observation of Cossins and Prosser (24) and Behan-Martin *et al.* (17) showing an adaptation of fatty acid composition and biophysical properties of brain synaptic membranes to environmental/body temperature over the evolutionary scale. In our experiments the extent of adjustment of fluidity was different with the two probes used. The anisotropy parameters measured by DPH–PA were almost identical at the respective body temperatures of the investigated species. Using TMA–DPH, similar anisotropy parameters were seen only with fish adapted to summer and winter temperatures, but with rat and bird nearly identical anisotropy parameters were obtained at 20 and 10°C, respectively. The above authors used DPH to describe the fluidity of these structures. This probe distributes randomly into the hydrophobic core of the membranes and thus reports the overall fluidity relationships. DPH–PA and TMA–DPH anchor to the lipid water interface (25,26). A major difference between DPH–PA and TMA–DPH is in their charges: the former is negatively and the latter is positively charged. R_{ss} values (Table 4) obtained for TMA–DPH were consistently lower than those for DPH–PA. TMA–DPH probes the membrane at the segment C_{8-10} while DPH–PA at the segment C_{10-12} (27). Since it is known that disorder increases toward the methyl end of fatty acyl chains (28) and DPH–PA reported more ordered structures than TMA–DPH, it is unlikely that this difference would come from the different vertical locations of the probes. Moreover it has been shown that both probes give identical R_{ss} values in identical environment (29). Thus it can be concluded, that TMA–DPH, due to its cationic nature, reported membrane regions rich in negatively charged phospholipids while DPH–PA, being repelled from this environment, reported regions where only neutral or positively charged phospholipids were present. Schroeder *et al.* (30) found that TMA–DPH reported more fluid structures in mice brain SPM and concluded that the outer leaflet is more fluid than the inner one reported by DPH–PA. Since DPH–PA and TMA–DPH reported idential fluidities in fish SPM, it is also possible that fish and mammalian synaptic plasma membranes differ in this respect.

In general, choline-containing glycerophospholipids make up 35-40% of phospholipids in synaptic plasma membranes (31–34) and dominate the outer leaflet (31). Over 90% of choline phosholglycerides is present in diacyl form. Diacyl phoshatidylethanolamines make up only 45–55% of the ethanolamine glycerophosholipids and the rest is present in alkyl-, or alkenylacyl form in vertebrate brains (35).

It is conceivable that alterations seen in composition of diacyl choline phosphoglycerides during the thermal evolution are at least partially involved in controlling membrane fluidity according to the temperature in certain regions of the synaptic plasma membranes. It can be expected that changes in disaturated components are one of the factors affecting the fatty acyl chain order and contribute to perfect compensation of membrane order for environmental/body temperature. An increase in disaturated species with increasing temperatures might render the membranes more ordered. Indeed, Hazel *et al.* (36) reported that mixing various amounts of 16:0/16:0 phosphatidylcholine to 16:0/22:6 phosphatidylcholine made the vesicles more ordered. Figure 1 shows that varying the proportion of 16:0/16:0 phosphatidylcholine in vesicles brought about a similar trend of alterations in structural order of fatty acyl chains as seen with native synaptic plasma membranes in the course of thermal evolution/adaptation at low measurement temperatures. However, the compensation of structural order in these artificial membranes was far below 100% observed for native synaptic plasma membranes. Since the R_{ss} vs. temperature curves were overlapping in case of "fish-like" (12% DPPC) and "rat-like" (20% DPPC) vesicles above 10°C, it is probable that DPPC alone, below a critical concentration, may not be involved in compensation of membrane structural order for temperature. The vast difference between the degree of compensation between the intact and artificial membranes made of synthetic phospholipids raises the question as to whether some other membrane components are involved in the observed homeoviscous response. Earlier, investigating the response by intact nerve cells given to changes of temperature under *in vitro* conditions using a spin labelled fatty acid, we found that a high degree of compensation was seen only with the intact cells and not with vesicles made of the extracted phospholipids (15).

The level of disaturated diacyl ethanolamine phosphoglycerides is low and does not change either seasonally in fish or during thermal evolution of vertebrates; thus this species might not be involved in controlling the biophysical properties of the membranes. The observation that the proportion of 1-monounsaturated, 2-polyunsaturated phosphatidylethanolamines decreases with increasing environmental/body temperature in synaptic plasma membranes is in line with our earlier observations on molecular species composition of livers

and brains of fish seasonally or evolutionarily adapted to contrasting temperatures (14–16). In a previous study we concluded that these phosphatidylethanolamines might play a role in the control of physical properties of membranes during thermal adaptation in fish liver (37). In fact we presented evidence showing that 18:1/22:6-, in contrast to 16:0/18:1 phosphatidylethanolamine, mixed to 18:0/22:6 phosphatidylcholine effectively decreased the acyl chain order in phospholipid vesicles using different *N*-(9-anthroyloxy) fatty acids and lowered the thermotropic phase transition temperature determined by electron spin resonance spectroscopic technique using 5-SASL (37). The ratio of 16:0/18:1 or 18:0/22:6 to 1-monounsaturated, 2 polyunsaturated phosphatidylethanolamines, increased from 0.19 and 0.95 in cold-adapted fish to bird, respectively, and from 0.96 and 13.46 in cold-adapted fish to bird, respectively, (Table 2). Thus a proper balance between certain molecular species in synaptic plasma membranes might contribute to the control of physical state according to environmental/body temperature.

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Regulation of Platelet-Activating Factor Receptor Expression in Human B Cells and B Cell Lines

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ABSTRACT: We extended our previous data regarding the modulation of human platelet-activating factor receptor (hPAF-R) expression on human B cell lines as well as normal B cells. First, we showed that hPAF-R mRNA was present in B cell lines expressing membrane hPAF-R, but was absent from cell lines devoid of hPAF-R. Second, enhanced hPAF-R membrane expression induced in IM9 line by IL4 was preceeded by hPAF-R mRNA accumulation that was detectable by 8 h and which peaked at 24 h. Similar results were observed for 10 nM platelet-activating factor treatment, which increased hPAF-R mRNA content up to 120% at 48 h, whereas hPAF-R membrane expression was up-regulated by 130%. Third, our data indicate that functional hPAF-R are expressed on resting, as well as on activated, B cells and that B cell activation is required for maintaining hPAF-R membrane and mRNA expression. Thus, in normal B cells, as well as in B cell lines, transcriptional regulation and/or messager stability control hPAF-R expression.

Lipids 31, 1051–1058 (1996).

Platelet-activating factor (PAF) is a potent phospholipid mediator of inflammatory, allergic, thrombotic, and shock reaction (1–3). Produced at the site of agression, PAF recruits and stimulates neutrophil, eosinophil, and macrophages. Accumulating data suggest that PAF may also act on lymphocytes and regulate their activation. Using 1-*O*-[^{3}H]octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine [^{3}H]PAF binding, we and others have demonstrated that several B cell lines expressed human platelet-activating factor receptor (hPAF-R) (4,5). We have also demonstrated that membrane hPAF-R expression is specifically enhanced by IL4 and by PAF itself (5). In addition, our data indicated that hPAF-R was expressed on resting as well as activated B cells, but was only present on activated T cells (5,6). Recently, cDNA cloning from various sources revealed that hPAF-R belongs to the β-adrenergic receptor family (7–10). hPAF-R displays a number of features that may characterize a new family of serpentine receptors (11). Using antibodies directed against two different parts of the extracellular region of human hPAF-R, two groups have definitively established hPAF-R expression in monocytes, granulocytes, neutrophils, and eosinophils (12,13). In contrast, they obtained conflicting results regarding hPAF-R expression on lymphocytes.

In the present work, we extended our previous data on B cell lines, showing that hPAF-R mRNA correlated with hPAF-R membrane expression. On normal B cells, the expression of functional membrane hPAF-R, as well as its modulation during cell activation, was analyzed.

MATERIALS AND METHODS

B cells and cell cultures. The final medium for cultures consisted of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 mM HEPES (Biochrom KG, Berlin, Germany). For culture in the presence of PAF, heat-inactivated FCS was depleted from acetylhydrolase activity by a brief acidic treatment as described previously (14,15). Cells were cultured in an humidified atmosphere containing 5% CO_2 in air at 37°C.

Nalm 6, Daudi, Raji, Ramos, and IM9 were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Rj2.2.5 and RM3 (two DR-deficient mutants of Raji) were provided by R. Accola (Verone, Italy) and A.F. Calman (San Franscico, CA), respectively (16,17). EBV1 and COR (a gift from B. Lisowska-Grospierre, Paris, France) cell lines were established by Epstein-Barr virus infection of normal peripheral B cells using the B95.8 (Epstein-Barr virus-producing Marmoset lymphoblastoid cell line) supernatant. Cell density was adjusted at 2–3 × 10^5 cells/mL, three times a week.

Human mononuclear cells were obtained from tonsils removed from children with chronic tonsillitis. Resting B cells were isolated from the E^- fraction by percoll gradient separation, as previously described (15). Normal resting B cells (1 × 10^6 cells/mL) were stimulated for 48 h by Staphylococcus Aureus Cowan 1 (SAC; 1:10^4, vol/vol; Calbiochem Corp., San Diego, CA), phorbol 12,13-dibutyrate (PDB; 1 ng/mL;

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Abbreviations: FCS, fetal calf serum; [^{3}H]PAF, 1-*O*-[^{3}H]octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; hPAF-R, human platelet-activating factor receptor; HSA, human serum albumin; lyso-PAF, 1-alkyl-*sn*-glycero-3-phosphocholine; MFI, mean fluorescence intensity; PAF, platelet-activating factor or PAF-acether, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PDB, phorbol 12,13-dibutyrate; PMA, 12-phorbol myristate 13-acetate; SAC, Staphylococcus Aureus Cowan.

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Sigma Chemical Co., St. Louis, MO) and ionomycin (800 ng/mL), phorbol 12-myristate 13-acetate (PMA; 5 ng/mL; Sigma), IL4 (400 U/mL, Genzyme, Cambridge, CA), or the combination of CD40 Ab (18) (from G28-5 hybridoma provided by E.A Clark, Seattle, WA) (1 μg/mL), and IL4. Cell count and viability (by trypan blue exclusion) were assessed at the time of the assay.

Binding assay of radiolabeled PAF. Synthetic PAF C_{18} (1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) and lyso-PAF (1-*O*-octadecyl-*sn*-glycero-3-phosphocholine) (Bachem, Bubendorf, Switzerland) were dissolved at 10 mM in ethanol (60%). The solutions were evaporated under nitrogen, redissolved at 100 mM in 0.9% NaCl containing 2.5 mg/mL fatty acid free–human serum albumin (HSA; Biomerieux, Lyon, France) and stored at −20°C. For further dilutions, the concentration of 2.5 mg/mL Fatty acid free–HSA was maintained. [^{3}H]PAF (sp. act. 9.25 MBq/mmol) was purchased from Amersham (Les Ulis, France).

[^{3}H]PAF binding to B cell lines was performed as previously described (5). In brief, cells (5×10^6 cells/mL) were incubated for 2 h at 4°C with [^{3}H]PAF (0.325–10 nM) with or without unlabelled PAF (500 nM). B cell associated-radioactivity was then measured on GF/C filters (Millipore, Ferrière, France) after vacuum filtration as described. The average number of hPAF-R/cell and the dissociation constant were quantified by Scatchard plot analysis of the specific binding data (5).

cDNA probes and Northern blot analysis. A 0.7 kb EcoRI cDNA fragment of hPAF-R (previously described in Ref. 12) was used as specific probe, whereas a 1.5 kb EcoRI cDNA probe of ribosomal RNA 18S (ATCC, Rockville, MD) was used for control hybridization. Probes were radiolabelled to a specific activity $>1 \times 10^8$ cpm/mg with [α^{32}P]dCTP (sp. act. >3000 Ci/mmol; Amersham) using a multiprime DNA labelling kit (Amersham).

Total RNA was extracted from cell preparations by guanidium isothiocyanate-phenol chloroform technique according to Chomczynski and Sacchi (19). Twenty μg of total RNA were electrophoresed through a 1% formaldehyde–agarose gel and blotted onto Hybond N^+ nylon membrane (Amersham). Specific hybridization was performed as previously described (19). Membranes were exposed to X-ray films (Hyperfilm MP; Amersham) with intensifying screens at −80°C for 48 h. Membranes were rehybridized with an 18*S* probe as control. Quantification of signal intensity was performed by a STARWISE image processing system (IMSTAR, Paris, France)

Immunofluorescence analysis. IOB4 (IgG1, CD19), ROM2 (IgG2a, CD14) monoclonal antibodies, FITC-conjugated goat $F(ab')_2$ anti-mouse IgG or FITC-conjugated goat $F(ab')_2$ anti-rabbit IgG were purchased from Immunotech (Marseille, France). Polyclonal hPAF-R antibody (12) was used at 1:500 dilution as defined by preliminary experiments. A polyclonal keyhole limpet hemocyanin antibody was used as control. Membrane hPAF-R expression was assessed by flow cytometry as previously described (5). Cell labelling was analyzed using a FACScan flow cytometer (Becton Dickinson, Mountainview, CA) using logarithmic scale for fluorescence. At least 5,000 events were analyzed after gating on viable cells for each histogram.

Measurement of $[Ca^{2+}]_i$ by flow cytometry. $[Ca^{2+}]_i$ mobilization was determined in B cell lines and normal B cells using fluorescent dye Indo-1 AM (Sigma) as previously described (13). Briefly, cells were washed twice in RPMI 1640, resuspended at 3×10^6 cells/mL in RPMI 1640 plus 10% FCS and loaded with 10 μM Indo-1 AM for 30 min at 37°C. Cells were washed twice in RPMI 1640 and allowed to equilibrate for an additional 30 min at 37°C. Cells were pelleted, resuspended at 2×10^6 cells/mL in Hank's balanced salt solution without Ca^{++} supplemented with 10 mM Hepes, and protected against light until use. Ca^{++} concentration was brought to 1.5 mM and cell suspensions were prewarmed at 37°C for 15 min prior to the assay. At the time of assay, PAF, lyso–PAF or Nacl–HSA were added to one mL of cell suspension and fluorescence signals recorded in an EPICS Prolile flow cytometer (Coulter, Hialeh, FL). Results are expressed as peak mean fluorescence intensity (MFI) (*y*-axis, linear scale) recorded every 60 s (*x*-axis).

RESULTS

Expression of membrane and mRNA hPAF-R in B cell lines. Binding of [^{3}H]PAF on several B cell lines is depicted in Figure 1A. As shown, Raji and IM9 expressed high numbers of hPAF-R, whereas no binding site was detected on Daudi and Nalm 6. Two HLA DR class II-deficient mutants of Raji, RM3 and Rj2.2.5, exhibited 3- and 10-fold less [^{3}H]PAF binding sites than did Raji. Compared to RM3, Ramos displayed similar number of sites. By contrast, two lymphoblastoid cell lines established from normal peripheral B cells were devoid of binding site. Scatchard analysis revealed, in all positive cell lines, a single class of high affinity hPAF-R with a kd value ranging from 1 to 4.5 nM (data not shown).

Northern blot analysis, (Fig. 1B) showed that hPAF-R mRNA was expressed as a single 3.8-4 kb transcript in Raji, IM9, RM3, and Rj2.2.5 cell lines, whereas no hPAF-R mRNA was detected in Daudi and Nalm 6. hPAF-R mRNA signal intensity was similar in B cell lines exhibiting high (Raji and IM9) or low number (Rj2.2.5 and RM3) PAF binding sites. This suggests that, in cell lines, hPAF-R membrane expression is regulated at transcriptional and post-transcriptional levels.

PAF modulation of hPAF-R mRNA expression. We next examined whether modulation of membrane hPAF-R expression induced by PAF itself (5) was regulated at a transcriptional level. Thus, IM9 B cells were cultured in 10% FCS acetylhydrolase-free medium with or without PAF for defined periods of time. An important increase of hPAF-R mRNA accumulation was observed with 10 and 100 nM PAF, and vehicle (NaCl–HSA) had no effect (Fig. 2A). For short-time exposure (6 h), 100 nM induced a maximal increase (87%) of hPAF-R mRNA level (Fig. 2B). For longer-time exposure, 10 nM was more efficient than 100 nM PAF in modulating hPAF-R mRNA level. For instance, at 48 h, the increase in mRNA content was 73 and 120% for 100 and 10 nM PAF, re-

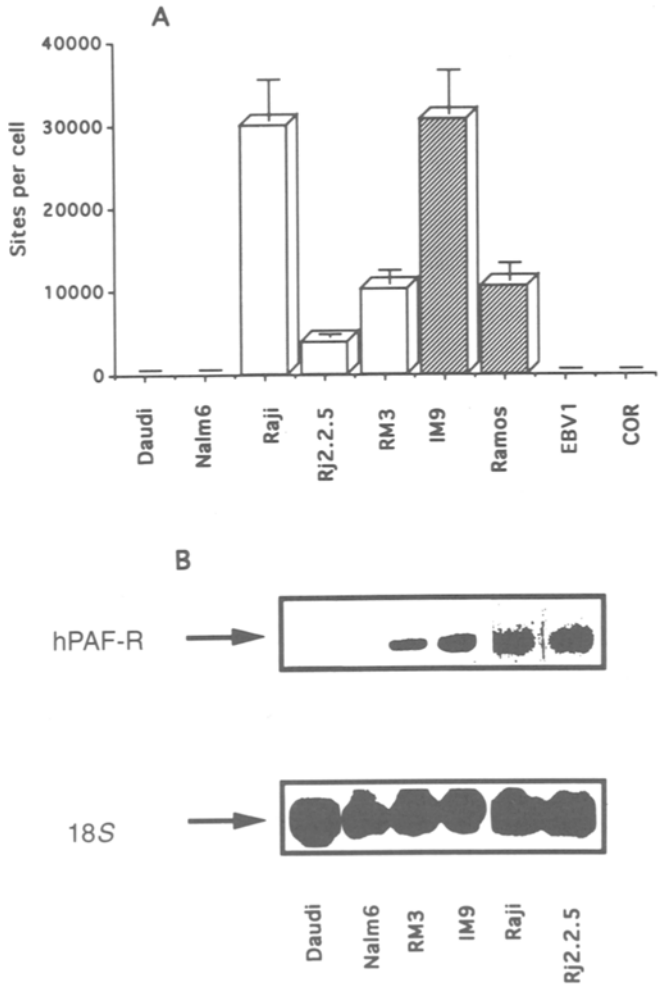


FIG. 1. Human platelet-activating factor receptor (hPAF-R) expression in human B cell lines. (A), binding on various B cell lines. Results are expressed as mean number of sites ± SD as determined by scatchard analysis. (B), Northern blot using ^{32}P-hPAF-R or ^{32}P ribosomal RNA 18*S* probes (20 µg/lane) (ATCC, Rockville, MD). One representative experiment out of six is shown.

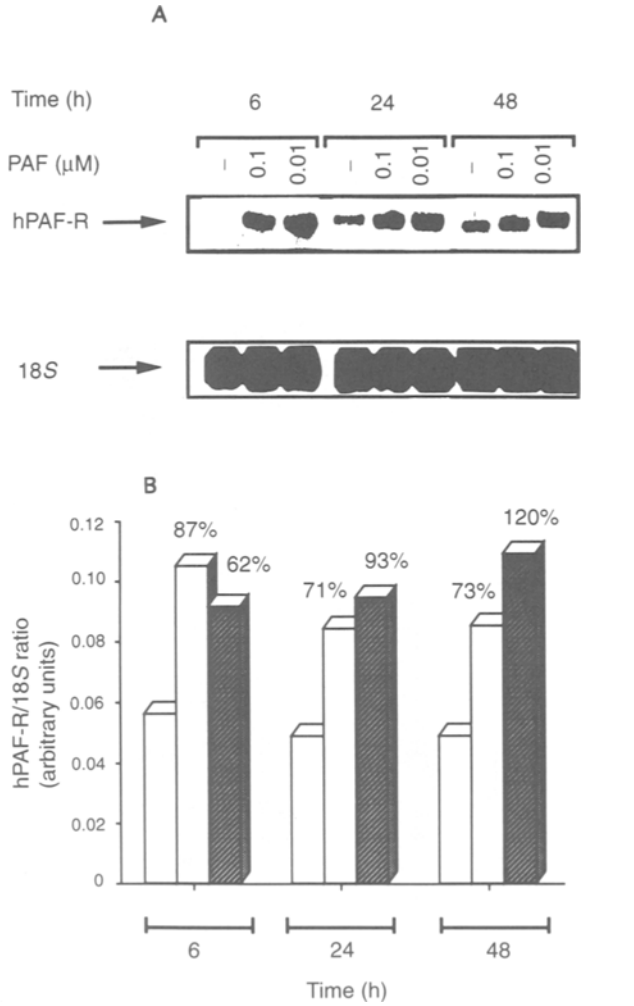


FIG. 2. hPAF-R mRNA expression is modulated by platelet-activating factor (PAF). IM9 cells (0.2×10^6/mL) were cultured for 6, 24, or 48 h in acetylhydrolase-free medium with 10 to 100 nM PAF or vehicle. (A), Northern blot analysis using ^{32}P-hPAF-R or ^{32}P ribosomal RNA 18*S* probes (20 µg/lane). Densitometric analysis of the blot is plotted in (B). One representative experiment out of three is shown. See Figure 1 for abbreviation and company source.

spectively. The increase in hPAF-R mRNA fits well with our previous observation, showing that treatment of IM9 cells with PAF (10 or 100 nM) up-regulated the membrane expression of its own receptor by about 130% (5).

IL4 modulation of hPAF-R mRNA expression. We have previously reported that IL4 up-regulated by about 150% the number of hPAF-R sites, without affecting the affinity (5). Here, kinetic studies on hPAF-R mRNA expression were performed after addition of IL4 (400 U/mL) or medium (Fig. 3). hPAF-R mRNA expression increased by 130% after 8 h, reached a maximum by 24 h (210%), and decreased thereafter (110 and 0% at 48 and 72 h, respectively).

Expression of hPAF-R mRNA in normal human B cells. We next investigated hPAF-R mRNA expression in normal B cells. Northern blot analysis was performed on RNA extracted from fresh resting B cells (resting) or from B cells cultured for 48 h with medium (none), SAC, IL4, PMA, or PDB plus ionomycin. Results in Figure 4A show that resting, but not medium-treated (48 h), B cells expressed hPAF-R mRNA. In activated B cells, hPAF-R mRNA level was similar (PDB plus ionomycin, IL4) or twofold higher (PMA, SAC) than that observed in resting B cells (Fig. 4B).

Resting and activated B cells express membrane hPAF-R. Using a polyclonal hPAF-R antibody, we also investigated membrane expression on normal B cells. Preliminary experiments indicated that this antibody stained IM9 and Raji, but not Daudi cell lines (data not shown). Figure 5 shows that all B cell populations were more than 91% $CD19^+$ and less than 3% $CD14^+$. hPAF-R expression recovered in 40 to 50% resting B cells (Fig. 5A) was lost after 48 h of culture in medium (data not shown). Stimulation with IL4 (Fig. 5B) increased the percentage of hPAF-R positive cells to 73% without changing the staining intensity (MFI equal to 64 vs. 52). Stimulation by SAC (Fig. 5C) or PDB plus ionomycin (Fig. 5D) had little effect on the percentage of positive cells (55

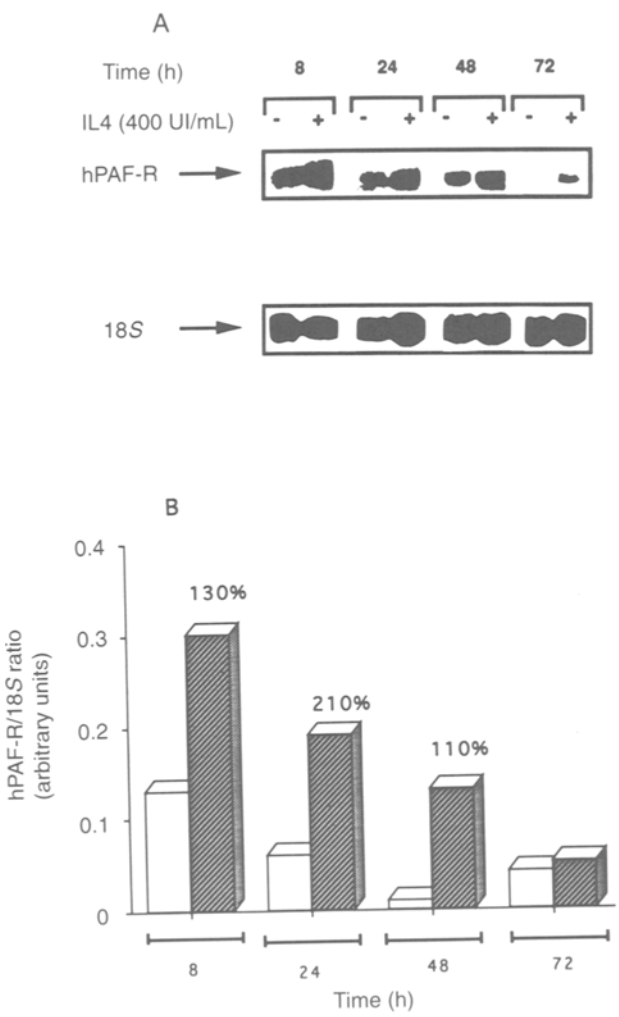


FIG. 3. hPAF-R mRNA expression is modulated by IL4. IM9 cells (0.2 × 10^6/mL) were cultured with IL4 (400 U/mL) or with medium for defined periods of time. (A), Northern blot analysis using using ^{32}P-hPAF-R or ^{32}P ribosomal RNA 18*S* probes (20 µg/lane). Densitometric scanning of the blot is computed in (B), One representative experiment out of two is shown. See Figure 1 for abbreviation and company source.

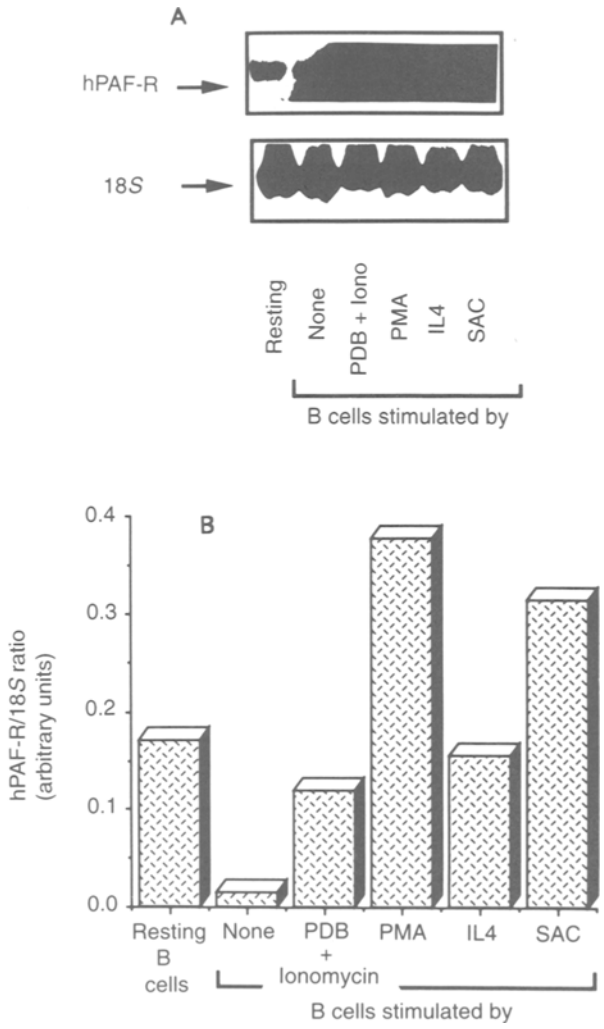


FIG. 4. hPAF-R mRNA expression in normal human B cells. B cells (1 × 10^6/mL) were cultured in medium (none) or stimulated with Staphylococcus Aureus Cowan (SAC) (1:10^4, vol/vol), IL4 (400 U/mL), 12-phorbol myristale 13-acetate (PMA) (5 ng/mL), or phorbol 12,13-dibutyrate (PDB) (1 ng/mL) plus ionomycin (Iono) (800 ng/mL) for 48 h before RNA extraction. RNA from fresh resting B (resting) cells was also extracted. (A), Northern blot analysis (20 µg/lane) using ^{32}P-hPAF-R or ^{32}P ribosomal RNA 18*S* probes. Densitometric scanning of the blot is computed in (B). One representative experiment out of three is shown. See Figure 1 for additional abbreviation and company source.

and 61% vs. 40%), but enhanced the staining intensity (MFI equals to 115 and 84 vs. 52, respectively). With PMA (Fig. 5E), both the percentage of positive cells and the staining intensity increased by twofold. Because B cell stimulation may also lead to a shift of CD19 MFI, we tried to quantify variations in hPAF-R expression by comparing the ratio between hPAF-R and CD19 MFI. The ratio for IL4-or PDB plus ionomycin-treated B cells was not different from that of resting B cells (0.26 and 0.27 vs. 0.25). For SAC- and PMA-treated B cells, the ratio increased up to 0.35 and 0.44, respectively.

Resting and activated B cells express functional hPAF-R. To investigate whether changes in membrane hPAF-R expression correlated with functional modifications, B cell populations activated by various agents were submitted to Ca^{2+} flux measurement. Results in Figure 6 show that both resting and activated B cells mounted a specific calcium response. Similar maximum values of MFI, ranging from 495 to 548, were obtained for B cells stimulated with SAC (Fig. 6C), PDB plus ionomycin (Fig. 6D) and PMA (Fig. 6E). For B cells cultured with IL4 alone (Fig. 6B) or CD40 antibody plus IL4 (Fig. 6F), the Ca^{2+} flux increased more rapidly and reached a maximum at 650 and 817 respectively. Resting (Fig. 6A) and IL4-treated B cells exhibited similar reactivity (maximum MFI equal to 614 and 650, respectively). As expected, addition of lyso-PAF did not induce Ca^{2+} fluxes.

DISCUSSION

Paf is a potent phospholipid mediator of inflammation produced by and acting on numerous cells. Accumulating data showed that monocytes and lymphocytes are important targets for PAF. On monocytes, PAF up-regulates the production of several cytokines

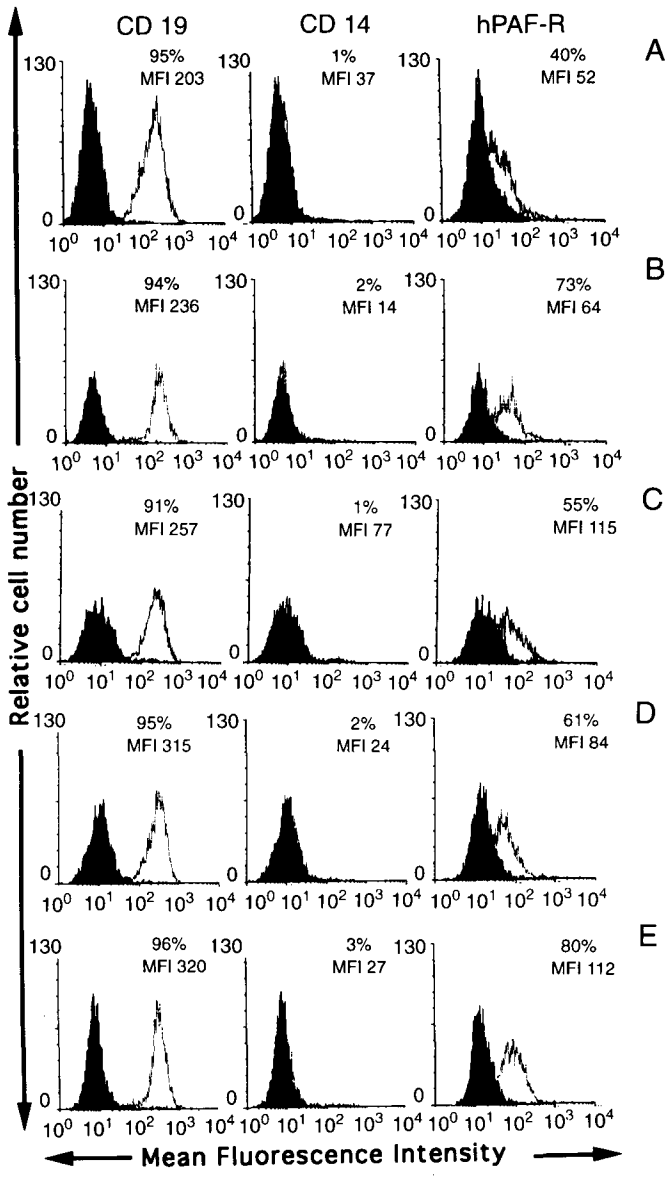


FIG. 5. Membrane hPAF-R expression on human B cells. B cells (1×10^6/mL) stimulated with IL4 (400 U/mL) (B), SAC (1:10^4, vol/vol) (C), PDB (1 ng/mL) plus ionomycin (800 ng/mL) (D), or PMA (5 ng/mL) (E), for 48 h were stained with CD19, CD14 mAbs, and polyclonal hPAF-R, as described in the Materials and Methods section. Fresh resting B cells were also analyzed (A). Mouse IgG and keyhole limpet hemocyanin polyclonal antibody were used as controls (dark histogram). Percent positive cells and mean fluorescence intensity (MFI) are indicated for each panel. One representative experiment out of two is shown. See Figure 1 for additional abbreviation.

(19–24) and PAF receptor expression is modulated by IFN_γ or PAF (25,26). On T lymphocytes, paf decreases CD2 and CD3 expression and differently regulates CD2- and CD3-mediated T cell activation (27–29). In addition, several investigators have demonstrated that PAF regulates important steps of human B cell activation, proliferation, and immunoglobulin production (30–34). hPAF-R expression is well established in B cell lines, but remains controversial on normal B lymphocytes (5,12,13).

In this report, we have shown that hPAF-R mRNA was present in B cell lines expressing membrane hPAF-R, but was absent from cell lines devoid of hPAF-R. By contrast, Raji 2.2.5 and RM3 expressed 3- and 10-fold less PAF binding sites than Raji without any difference in hPAF-R mRNA contents. Moreover, IL4 or exogenous PAF modulated hPAF-R mRNA levels in IM9 and Raji cell lines, but not in Nalm 6 or Daudi. After IL4-treatment of IM9 cells, hPAF-R mRNA increased by 130% at 8 h and maximal expression was observed by 24 h (210%). At 24 h, a 150% increase of membrane hPAF-R number occurs upon IL4 treatment (5). Thus, IL4 may control IgE production in B cells by two mechanisms: directly, by controling isotype switching; and indirectly, by favoring hPAF-R expression and thus, responsiveness to PAF. On monocytes, IFNγ also upregulated hPAF-R mRNA and membrane expression (25).

In addition to IL4, PAF itself also enhanced hPAF-R mRNA expression on IM9 cells. Although hPAF-R mRNA accumulation induced by 10 and 100 nM PAF followed different kinetics, a marked increase from 73 to 120% was observed at 48 h. At that time, 10 nM PAF induced an increase of similar magnitude for hPAF-R membrane and mRNA expression (130 and 120%, respectively). Up-regulation of hPAF-R expression, following exposure to PAF, contrasts with homologous desensitization of hPAF-R previously reported, for instance, in human platelets (35,36) or Kupffer cells (37). Depending on the nature of cell target, different mechanisms, including hPAF-R loss, conformational changes, or uncoupling of the receptor, have been envisioned for explaining desensitization (38,39). Additionally, hPAF-R up- or down-regulation may be dependent on the concentration of PAf used. In this regard, we previously observed dual biological effects of PAF on normal activated B cells—1 and 100 nM PAF increased cell proliferation, whereas 1 to 10 μM inhibited it. In Raji cells which express 10- to 20-fold more hPAF-R than normal B cells, 1 μM PAF increased cell proliferation and [^{3}H]PAF binding (5). In human monocytes, which constitutively express more hPAF-R membrane than B cells (12), 10 nM to 1 μM PAF also induced a twofold increase in hPAF-R mRNA expression (26). At the present time, the biological relevance of the PAF-mediated upregulation of its own receptor remains open to discussion. One likely and interesting possibility is that triggering of hPAF-R by its ligand may lower the threshold for PAF responsiveness. The modulation of hPAF-R expression may be relevant to the regulation of the immune response by providing a dynamic and on-demand regulation of various cellular responses, depending on local PAF concentration.

Low levels of [^{3}H]PAF binding on normal B cells led us to examine membrane, as well as mRNA hPAF-R expression. All resting B cells tested expressed 10- to 20-fold lower PAF mRNA than IM9 (data not shown). The loss of hPAF-R mRNA signal after 48 h of culture in medium alone suggests that B cell activation was required for maintaining its expression. By immunofluorescence, membrane hPAF-R expression was detected on 40 to 50% of resting B cells. After activation,

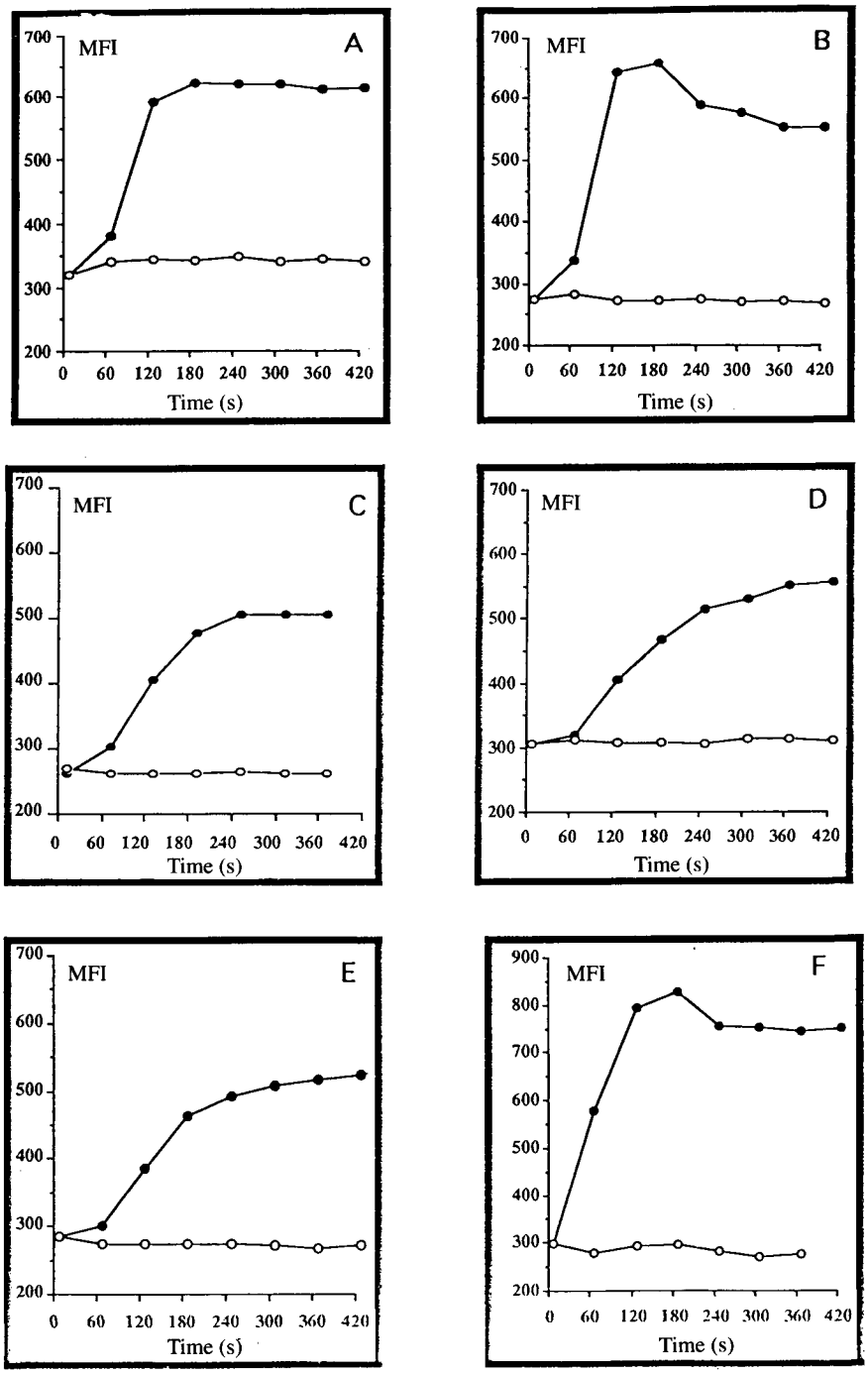


FIG. 6. PAF induces $[Ca^{2+}]_i$ mobilization in resting and activated B cells. B cells were activated for 48 h with IL4 (400 U/mL) (B), SAC (1:10^4, vol/vol) (C), PDB (1 ng/mL) plus ionomycin (800 ng/mL) (D), PMA (5 ng/mL) (E), or CD40 (1 μg/mL) plus IL4 (400 U/ml) (F), Resting (A) and activated B cells were loaded with Indo-1/AM. Changes in $[Ca^{2+}]_i$ were recorded by flow cytometry. Results are expressed as change in MFI (linear scale) after stimulation with PAF (●) or lysoPAF (○). One representative experiment out of two is shown. See Figures 2 and 5 for abbreviations.

the percentage of positive cells and/or the staining intensity increased with a maximal induction observed in the presence of PMA or SAC. Reactivity of resting or activated B cells was not due to residual monocytes as similar results were obtained using B cells purified with CD19 dynabeads (data not shown). Thus, these results indicate that B cell activation plays a major role in expression and conformational changes of the hPAF-R. By contrast to our study and the one by Müller *et al.* (12), Simon *et al.* (13) reported the lack of hPAF-R on human resting B cells. It is worth mentioning that we did use an anti-

body directed against residues 164 to 173, which included a part of the second extracellular loop of the hPAF-R. This region which also contained the unique *N*-glycosylation site and one of the two conserved cystein residues is required for stability of ligand binding pocket (33). In contrast, Simon *et al.* (13) used an antibody raised against the NH2-terminal region, not essential for ligand binding. As both antibodies detected the presence of hPAF-R in IM9 and Ramos, one can speculate that apparent differences in hPAF-R expression is due to a special conformation of the receptor on resting B cells.

Although most stimuli increased transcription and sustained hPAF-R expression in B cells, some of them also favored functional responses. Indeed, stimulation with CD40 antibody plus IL4 raised up the capacities of B cells to respond to PAF. This result is particularly important as IL4 and the signal delivered through CD40/CD40L interactions play major roles in IgE production and memory B cell generation (40, 41). Whether CD40 triggering acts directly on hPAF-R expression, conformation, or indirectly favors PAF response through TNFα production and/or NF-KB activation remains to be established.

In summary, ours results showed that: (i) spontaneous hPAF-R expression in B cell lines seems essentially regulated at a transcriptional level; (ii) IL4 and PAF control hPAF-R expression by acting at a transcriptional level and/or by stabilizing hPAF-R mRNA; (iii) functional hPAF-R are expressed on resting and activated B cells and (iv) B cell activation may regulate hPAF-R expression, conformation, and function. The latter data and the observation that PAF regulates cytokine production suggest that PAF is an important modulator of B cell response and differentiation.

ACKNOWLEDGMENTS

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The Effects of Lidocaine and Hypoxia on Phospholipid Biosynthesis in the Isolated Hamster Heart

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ABSTRACT: In this study, the effects of lidocaine and hypoxia on the biosynthesis of phospholipids in the hamster heart were examined. Hamster hearts were perfused with [1,3-^{3}H]glycerol under normal and hypoxic conditions, and in the absence or presence of 0.5 mg/mL lidocaine. After perfusion, the radioactivity incorporated into the various phospholipid fractions was determined. With the exception of phosphatidylcholine, the synthesis of phospholipids was generally stimulated by lidocaine perfusion. In contrast, hypoxia caused a general decrease in phospholipid biosynthesis which was partially restored by lidocaine. ATP and CTP levels were severely reduced under hypoxic conditions, but their levels were not restored by lidocaine treatment. The activities of enzymes for phospholipid synthesis were determined under the various perfusion conditions. The activity of phosphatidic acid phosphatase was elevated by lidocaine and decreased by hypoxic treatment. The activity of CTP:phosphatidic acid cytidylyltransferase was increased under hypoxia, with or without lidocaine. Despite the reduction in phosphatidylcholine biosynthesis, no change in the activity of cytidine diphosphocholine (CDPcholine):diacylglycerol cholinephosphotransferase was detected following lidocaine or hypoxic perfusion. However, enzyme activity was inhibited by the presence of lidocaine in the assay mixture. Our results indicate that the reduction in phospholipid biosynthesis under hypoxic conditions was caused mainly by diminishing high-energy nucleotide levels. The enhancement of phospholipid biosynthesis by lidocaine appeared to be mediated in part by modulation of enzyme activities.

Lipids 31, 1059–1067 (1996).

Phospholipids are the major lipid component of biological membranes (1). In mammalian tissues including the heart, the appropriate content and composition of phospholipids are important for the maintenance of cellular integrity and proper membrane function. In addition, alterations in membrane phospholipids may affect the conduction of electrical signals across the cardiac membrane (2). Hence, the biosynthesis and catabolism of phospholipids are under rigid control. The pathways for the synthesis of the major phospholipids in the heart have been largely elucidated (3,4), but the mechanisms controlling these metabolic pathways have not been fully explored.

Perfusion of the hamster heart *ex vivo* in the Langendorff mode is a useful model to study the control of phospholipid metabolism in the isolated organ. When the hamster heart was perfused under hypoxic conditions or in the presence of amphiphilic compounds such as lidocaine or methyl-lidocaine, alterations in phospholipid biosynthesis were detected (5–7). Perfusion under hypoxia caused a large reduction of the ATP and CTP levels in the heart, with a concomitant decrease in the incorporation of radioactive choline into phosphatidylcholine (5). However, the effects of hypoxia on other phospholipid groups were not known. When the hamster heart was perfused with lidocaine, increases in the synthesis of lysophosphatidic acid, phosphatidic acid, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol were observed (7). In contrast, the synthesis of phosphatidylcholine was decreased under identical conditions. Further investigation revealed that the activities of key enzymes responsible for phospholipid biosynthesis were modulated by lidocaine. Because lidocaine has been employed routinely as a therapeutic agent for the treatment of cardiac arrhythmias, it would be of interest to study its action on phospholipid biosynthesis under hypoxic conditions. In the present study, the effect of hypoxia on the biosynthesis of phospholipids in the heart and the effect of lidocaine on phospholipid biosynthesis during hypoxia were examined.

MATERIALS AND METHODS

Materials. Lidocaine hydrochloride was obtained from Astra Pharmaceutical Products (Mississauga, Ontario, Canada). [1,3-^{3}H]Glycerol (3.1Ci/mmol), [1-^{14}C]oleoyl-coenzyme A (CoA) (54 mCi/mmol), [1-^{14}C]palmitoyl-CoA (47.8 mCi/mmol), [5-^{3}H]CTP (58 mCi/mmol), *myo*-[2-^{3}H]inositol (18.2 Ci/mmol), and [^{3}H]acetic anhydride (0.5 Ci/mmol) were

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Abbreviations: CDPcholine, cytidine diphosphocholine; CDP-diacylglycerol, cytidine diphosphate diacylglycerol; CDPethanolamine, cytidine diphosphoethanolamine; CoA, coenzyme A; NEN, *N*-ethylmaleimide; TLC, thin-layer chromatography.

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obtained from Amersham International (Oakville, Ontario, Canada). Cytidine diphosphocholine (CDP[methyl-^{14}C]choline) (58 mCi/mmol) and cytidine diphosphoethanolamine (CDP[1,2-^{14}C]ethanolamine) (97 mCi/mmol) were obtained from the NEN Division of Dupont Co. (Dorval, PQ, Canada). All lipid products were obtained from Serdary Laboratories (London, Ontario, Canada). BCl_3-methanol reagent was obtained from Supelco Canada (Mississauga, Ontario, Canada). All other chemicals, solvents, and biochemicals were of reagent grade and were obtained from the Canlab Division of Baxter Company (Winnipeg, Manitoba, Canada) or Sigma Chemical Company (St. Louis, MO).

Perfusion of the isolated hamster heart. Syrian Golden hamsters weighing 120 ± 20 g were used throughout the study. Isolated hearts were perfused for 60 min in the Langendorff mode (cannulation *via* the aorta) with Krebs-Henseleit buffer containing 1.0 mM [1,3-^{3}H]glycerol (3 μCi/μmol), saturated with a gas mixture composed of 5% CO_2 and either 95% O_2 (control) or 95% N_2 (hypoxia). In some experiments, 0.5 mg/mL lidocaine was added to the perfusate. The viability of the heart during perfusion was monitored by electrocardiac recording. An electrode was placed on the aorta and another electrode was immersed in the buffer solution bathing the apex of the heart. Perfusion of the heart under hypoxic conditions from 30 to 60 min caused some electrophysiological and morphological changes that were reversible upon reoxygenation (data not shown). Hypoxic exposure of the heart in excess of 60 min caused irreversible damage to the cardiac tissue (5).

Determination of uptake and incorporation of radioactive glycerol. After perfusion, 10 mL of fresh Krebs-Henseleit buffer was injected through the cannula to remove residual radioactive perfusate. The hearts were homogenized in 20 mL chloroform/methanol (1:1, vol/vol), and the insoluble material was sedimented by centrifugation. An aliquot of the supernatant was taken for determination of radioactivity by scintillation counting. A 0.25 M KCl solution and chloroform were added to the supernatant to cause phase separation, and samples of the aqueous and organic phases were taken for radioactive determination. The extraction efficiency of labeled phospholipids by this method was 88–90% and was not affected by lidocaine. The phospholipid and neutral lipid classes in the organic phase were separated by thin-layer chromatography (TLC) in the following solvent systems. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine fractions were separated in a solvent containing chloroform/methanol/15 M aqueous NH_3/water (35:15:2:1, by vol). The solvent used for the separation of phosphatidic acid and lysophosphatidic acid contained chloroform/methanol/HCl (87:13:0.2, by vol) (8). Cytidine diphosphate (CDP-)diacylglycerol was separated in a solvent containing chloroform/methanol/acetic acid/water (52:20:7:3, by vol) (9). The solvent for the separation of diacylglycerol contained light petroleum (bp 35–60°C)/diethyl ether/acetic acid (60:40:1, by vol). After visualization by iodine staining, the bands on the TLC plate corresponding to authentic standards were scraped into scintillation vials and their radioactivities were determined.

Lipid determinations. The major phospholipids separated by TLC were quantified by lipid phosphorus determination according to the procedure of Bartlett (10). For the determination of phosphatidylinositol and phosphatidylserine, the procedure of Rouser *et al.* (11) was used. For the determination of lysophosphatidic acid and phosphatidic acid, the procedure of Zhou and Arthur (12) was employed. Using phosphatidylcholine as a standard, the result obtained by the procedure of Rouser *et al.* (11) or Zhou and Arthur (12) was found to be comparable to that obtained by the Bartlett method (10). Diradylglycerol, of which 90% is in diacyl form (13), was isolated from the lipid extract by TLC, and the acyl groups were converted to the respective methyl esters by reaction with BCl_3-methanol (14). The fatty acid methyl esters were quantified by gas–liquid chromatography using a Shimadzu (Tokyo, Japan) Mini-2 gas–liquid chromatograph equipped with 15% DEGS (on 80/100 Chromosorb W/AW) columns. Heptadecanoic acid methyl ester was used as a standard for quantification.

Determination of ATP and CTP levels. ATP and CTP levels in the heart were determined as described earlier (5). After perfusion, hearts were stored temporarily in liquid nitrogen, and then homogenized in 6 mL chloroform/methanol (2:1, vol/vol). A small amount of labeled CTP was added to the homogenates for the calculation of yield. Water (3 mL) was added to the homogenate to cause phase separation. The aqueous phase was removed and the organic phase was re-extracted twice with 3 mL of water. The pooled aqueous phase extract was concentrated by evaporation under reduced pressure, and the nucleotide content in each sample was analyzed by high-pressure liquid chromatography using an Ultrasphere-ODS reversed-phase column (Beckman, Mississauga, Ontario, Canada). Nucleotides were eluted from the column with 0.03 M KH_2PO_4 containing 0.02 M tetrabutylammonium phosphate in 19% acetonitrile, pH 2.65.

Determination of cytidine diphosphocholine levels. Samples were prepared in the same manner as those used for the analysis of ATP and CTP. The recovery of CDPcholine was calculated from a small amount of CDP-[methyl-^{14}C]choline added to the tissue homogenate. The concentrated aqueous sample was dissolved in 10 mL water and applied to a Dowex AG1-X8 (Sigma-Aldrich Canada, Mississauga, Ontario, Canada) (OH^- form) column (1 cm × 30 cm). Choline was removed from the column by 60 mL methanol/water (1:1, vol/vol) followed by 10 mL water, and CDPcholine and phosphocholine were co-eluted with 200 mL 0.4 M NH_4HCO_3. The fraction containing the CDPcholine and phosphocholine was evaporated under reduced pressure and redissolved in 5 mL water. The sample was applied to a Norite A (Sigma-Aldrich Canada)/celite (1:1, vol/vol) column (1 cm × 5 cm). Phosphocholine was eluted from the column with 20 mL water and 15 mL 2% ethanol, and CDPcholine was eluted with 30 mL 40% ethanol containing 1% NH_4OH. The CDPcholine fraction was treated with 10 units of alkaline phos-

phatase and 10 units of *Crotalus adamanteus* phosphodiesterase (type III) to yield choline. The choline in this sample was purified by extraction into 3 mL heptan-3-one containing tetraphenylboron (10 mg/mL). The choline in the heptan-3-one was back-extracted with 0.5 mL 0.4 M HCl followed by a second extraction with 3 mL 1.0 M HCl. The HCl extracts were combined and lyophilized, and the choline was determined by the quantitative conversion of choline into [γ-^{32}P]phosphocholine (15).

Determination of enzyme activities. After perfusion, hearts were homogenized in a buffer containing 0.25 M sucrose and 10 mM Tris/HCl (pH 7.4). The postnuclear fraction was obtained by centrifugation at 2000 × *g* for 10 min. The postmitochondrial fraction was obtained by centrifugation of the postnuclear fraction at 12,000 × *g* for 15 min. The cytosolic and the crude microsomal fractions were obtained by centrifugation of the postmitochondrial fraction at 100,000 × *g* for 60 min. The resulting supernatant was designated as the cytosolic fraction, while the microsomal pellet was suspended in 10 mM phosphate buffer (pH 7.4). The protein contents of the various fractions were determined by the method of Lowry *et al.* (16). Enzyme activities were assayed in the subcellular fractions as described in the following sections.

Acyl-CoA:glycerol-3-phosphate and acyl-CoA:lysophosphatidic acid acyltransferases. The activities of acyl-CoA:glycerol-3-phosphate and acyl-CoA:lysophosphatidic acid acyltransferases were determined in the postnuclear fraction by the procedure of Batenburg *et al.* (17). For the acylation of glycerol-3-phosphate, the assay mixture consisted of 100 mM Tris/HCl (pH 7.5), 1.5 mM glycerol-3-phosphate, 90 mM sucrose, 0.5 mg of bovine serum albumin, 1 mM dithiothreitol, 40 μM [1-^{14}C]oleoyl-CoA (4000 dpm/nmol) and approximately 0.2 mg protein in a volume of 500 μL. The reaction was initiated by addition of the postnuclear fraction, incubated at 30°C for 30 min, and terminated by addition of 3 mL chloroform/ methanol (2:1, vol/vol). A solution containing 0.5 M NaCl was then added to the mixture to cause phase separation. The presence of a high salt concentration in the aqueous phase facilitated the recovery of lysophosphatidic acid in the organic phase. The lipid fractions in the organic phase were separated by TLC, and the distribution of radioactivity in the lipid fractions was scanned by a Bioscan System 200 Imaging Scanner (Interscience Inc., Markham, Ontario, Canada). The vast majority (>85%) of the labeling was found in the lysophosphatidic acid fraction. The radioactivity in this fraction was determined by scintillation counting.

For the acylation of lysophosphatidic acid, the reaction was carried out under the same conditions, except that *sn*-glycerol-3-phosphate was replaced with 0.2 mM 1-palmitoyl-*sn*-glycerol-3-phosphate (17). The labeled phosphatidic acid formed in the reaction was separated by TLC and the radioactivity in that fraction was determined.

Phosphatidic acid phosphatase assay. Phosphatidic acid phosphatase activity was assayed in the cytosolic and microsomal fractions by determining the production of 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycerol from the corresponding phosphatidic acid (18). The labeled phosphatidic acid was obtained from 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycerophosphocholine by phospholipase D hydrolysis (19). The assay mixture (total volume 100 μL) contained 100 mM Tris/HCl (pH 7.4), 1 mM dithiothreitol, 0.2 mg bovine serum albumin, 0.6 mM 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycerol-3-phosphate (2000 dpm/nmol), 0.4 mM phosphatidylcholine, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 0.75 mM oleic acid, and approximately 50 μg protein. The phosphatidic acid and phosphatidylcholine substrates were dispersed by sonication prior to their addition to the assay mixture. The cytosolic fraction was preincubated with oleic acid for 20 min at 37°C prior to the assay. The reaction was initiated by the addition of the appropriate subcellular fraction to the assay mixture and incubated at 37°C for 60 min. The reaction was terminated by the addition of 2 mL chloroform/ methanol (2:1, vol/vol). After phase separation, the labeled diacylglycerol in the organic phase was separated by TLC and the radioactivity in that fraction was determined.

CDPcholine:1,2-diacylglycerol cholinephosphotransferase and CDPethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase assays. CDPcholine:1,2-diacylglycerol cholinephosphotransferase and CDPethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase activities were assayed in the microsomal fraction by determining the transfer of CDP-[^{14}C]choline or CDP-[^{14}C]ethanolamine, respectively, to diacylglycerol (20,21). The reaction mixture for the cholinephosphotransferase assay (200 μL) contained 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.4 mM CDP-[^{14}C]-choline (1.0 μCi/μmol), 0.2 mM diacylglycerol (prepared in 0.015% Tween 20), final pH 8.5, and 50 μg protein. The reaction was initiated by the addition of labeled CDPcholine, and the mixture was incubated at 37°C for 15 min. The reaction was terminated by the addition of 3 mL chloroform/methanol (2:1, vol/vol), and water was added to cause phase separation. The phosphatidylcholine in the organic phase was separated by TLC and the radioactivity in that fraction was determined.

The reaction mixture for ethanolaminephosphotransferase (200 μL) contained 10 mM Tris-HCl (pH 8.5), 10 mM $MnCl_2$, 1 mM EDTA, 0.2 mM CDP-[^{14}C]ethanolamine (1.0 μCi/μmol), 0.2 mM diacylglycerol (prepared in 0.015% Tween 20), final pH 8.5, and 50 μg protein. The reaction was initiated by the addition of labeled CDPethanolamine, and the mixture was incubated at 37°C for 15 min. The reaction was terminated by the addition of 3 mL chloroform/methanol (2:1, vol/vol), and water was added to the mixture to cause phase separation. The phosphatidylethanolamine in the organic phase was separated by TLC and the radioactivity in that fraction was determined.

CTP:phosphatidic acid cytidylyltransferase assay. CTP:phosphatidic acid cytidylyltransferase activity was assayed in the microsomal fraction using [5-^{3}H]CTP and phosphatidic acid as substrates (22). The reaction mixture contained 50 mM Tris/maleate (pH 6.5), 20 mM $MgCl_2$, 15 mM

Triton X-100, 1.0 mM [5-^{3}H]CTP (10,000 dpm/nmol), 0.5 mM phosphatidic acid, and 50 μg protein. The reaction was initiated by addition of the microsomal fraction and the mixture was incubated at 37°C for 15 min. The reaction was terminated by the addition of 2.5 mL 0.1 M HCl in methanol. After cooling, 5 mL chloroform was added to the mixture. The chloroform/methanol mixture was washed three times with 10 mL 2 M $MgCl_2$ (23), and the total radioactivity in the organic phase was determined. TLC analysis of the organic phase showed that over 98% of the radioactivity was located in the CDP-diacylglycerol fraction.

Phosphatidylinositol synthase assay. Phosphatidylinositol synthase activity was assayed in the microsomal fraction by determining the transfer of *myo*-[^{3}H]inositol to CDP-dipalmitoylglycerol (24). The reaction mixture (total volume 100 μL) contained 100 mM Tris-HCl (pH 7.5), 1 mM EGTA, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 0.2% Triton X-100, 0.1 mM CDP-dipalmitoylglycerol, 0.1 mM *myo*-[^{3}H]inositol (5×10^5 dpm/nmol), and 50 μg protein. The reaction mixture was incubated at 37°C for 30 min, and the reaction was terminated by addition of 1 mL chloroform/methanol/HCl (100:100:1, by vol) and 0.4 mL 0.9% KCl. After phase separation, the labeled phosphatidylinositol in the organic phase was separated by TLC and the radioactivity in that fraction was determined.

Serine base exchange enzyme assay. Serine base exchange enzyme activity was assayed in the microsomal fraction by determining the production of radioactive phosphatidylserine from [^{3}H]serine and phosphatidylethanolamine (25). The reaction mixture (total volume 250 μL) contained 10 μM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.3), 0.8 μmol phosphatidylethanolamine, 20 nmol [^{3}H]serine (78,000 dpm/nmol), 6 μmol $CaCl_2$, 50 μg bovine serum albumin, and 50 μg protein. The reaction was carried out at 37°C for 15 min, and terminated by 2 mL chloroform/methanol (2:1, vol/vol). After phase separation, the phosphatidylserine in the organic phase was separated by TLC and the radioactivity in that fraction was determined.

Statistical analysis. Statistical significance was determined by analysis of variance, followed by two-tailed independent Student's *t*-tests. The level of significance was defined as $P < 0.05$.

RESULTS

Perfusion of hamster hearts. Hamster hearts were perfused with Krebs-Henseleit buffer containing 1 mM [1,3-^{3}H]glycerol. The buffer was saturated with either 95% O_2/5% CO_2 or 95% N_2/5% CO_2 prior to and during perfusion. Analysis of oxygen content in the perfusate showed that the $_PO_2$ in the oxygen-saturated buffer (for controls) was over 500 mm Hg, whereas the $_PO_2$ in the N_2-saturated buffer (for hypoxic perfusion) was less than 40 mm Hg. The $_PCO_2$ in all buffers was 35–40 mm Hg, which produced a pH value of 7.40 ± 0.05. The uptake of labeled glycerol by the hamster heart was found to be linear under normoxic and hypoxic conditions for up to 60 min of perfusion (6,7, and unpublished data). Hearts perfused under hypoxic conditions for 60 min displayed an abnormal electrocardiogram which was readily reversible upon re-oxygenation (5). Some degree of mitochondrial swelling at 60 min of hypoxic perfusion was observed by electron microscopy, and the swelling was found to be reversible upon re-oxygenation (5). Hypoxic exposure beyond 60 min caused irreversible electrophysiological and morphological damage (26,27). Hence, hearts were perfused under hypoxic conditions for 60 min or less in all experiments.

Modulation of phospholipid biosynthesis by lidocaine and hypoxia. In this study, hamster hearts were perfused under normoxic or hypoxic conditions in the absence or presence of 0.5 mg/mL lidocaine. After perfusion, the heart was homogenized and the total uptake of labeled glycerol was determined. The uptake of glycerol was markedly increased (88%) when the heart was perfused with 0.5 mg/mL lidocaine under normoxic conditions, and the majority of the increase in radioactivity was found in the organic phase (Fig. 1). Analysis of the organic phase by TLC revealed that there were increases in the labelings of lysophosphatidic acid (181%), phosphatidic acid (135%), CDP-diacylglycerol (153%), phosphatidylinositol (75%), phosphatidylserine (90%), phosphatidylethanolamine (157%), and diacylglycerol (258%) (Fig. 2). In contrast, the labeling of phosphatidylcholine was significantly decreased (40%).

When the heart was perfused under hypoxic conditions, the uptake of labeled glycerol was reduced (47%), and the majority of the reduction in radioactivity was confined to the organic phase (Fig. 1). Analysis of the organic phase by TLC showed general reductions in radioactivity associated with all lipid fractions (Fig. 2). When 0.5 mg/mL lidocaine was present in the hypoxic buffer, the reduction in total glycerol uptake became less severe (24%). This partial restoration of labeled glycerol uptake was also reflected in radioactivity found in the organic phase. Analysis of labelings of lipid fractions by TLC revealed that all phospholipid fractions except phosphatidylcholine were restored to their control values by lidocaine (Fig. 2). The reduction of radioactivity incorporated into phosphatidylcholine during hypoxic perfusion was not altered by the presence of lidocaine.

Alterations in the labeling of the lipid fractions might arise from changes in the pool sizes of any labeled glycerol-containing lipid precursors. Hence, the pool sizes of phospholipid and diacylglycerol fractions in the heart perfused under hypoxic conditions or in the presence of lidocaine were determined. As depicted in Table 1, phospholipid and diacylglycerol contents in the heart were not affected by hypoxic perfusion or lidocaine treatment. We have shown previously that the pool of CDP-diacylglycerol in the heart was not affected by lidocaine treatment (7). Recently, the cardiac CDP-diacylglycerol pool was found to remain unchanged under hypoxia (28). Thus, the observed alterations in the labeling of the cardiac phospholipids did not appear to arise from changes in their pool sizes or those of their precursors.

Effect of hypoxia on nucleotide contents. Because ATP and CTP are required for the synthesis of all phospholipids,

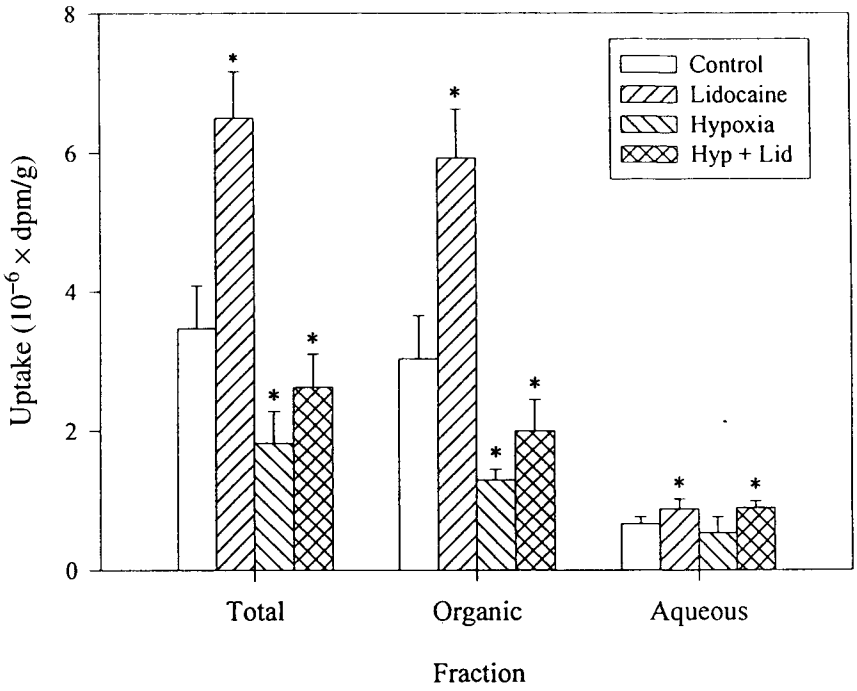


FIG. 1. The effects of lidocaine and hypoxia on the uptake of labeled glycerol in the hamster heart. Hamster hearts were perfused with Krebs-Henseleit buffer containing 1.0 mM [1,3-^{3}H]glycerol (3 μCi/μmol) for 60 min. The buffer was saturated with either 95% O_2 (control) or 95% N_2 (hypoxic), and contained either 0 or 0.5 mg/mL lidocaine. Tissue extraction and phase separation were performed as described in the Materials and Methods section, and samples were taken for determination of radioactivity. The results are expressed as means ± SD. The number of experiments was as follows: control, $n = 13$; lidocaine, $n = 11$; hypoxia, $n = 4$; hypoxia + lidocaine, $n = 3$. *$P < 0.05$ compared with control.

changes in levels of these nucleotides may affect the rate of phospholipid biosynthesis. Hence, the levels of ATP and CTP in the hamster heart perfused under hypoxia or with lidocaine were determined. As shown in Table 2, the levels of ATP and CTP were not affected by lidocaine perfusion, but were significantly reduced in the hypoxic heart. The presence of lidocaine during hypoxic perfusion did not cause any significant change in the levels of these nucleotides.

Because CDPcholine is a key metabolite for the synthesis of phosphatidylcholine, its level in the heart perfused under hypoxic conditions or in the presence of lidocaine was determined. Our results show that the CDPcholine content in the

TABLE 1
Phospholipid and Neutral Lipid Contents in the Hamster Hearts[a]

	Content (μmol lipid P/g wet weight of heart)			
Fraction	Control $n = 9$	Lidocaine $n = 6$	Hypoxia $n = 3$	Hypoxia + lidocaine $n = 3$
Lysophosphatidic acid	0.06 ± 0.01 (6)	0.09 ± 0.01 (3)	0.04 ± 0.01	0.06 ± 0.01
Phosphatidic acid	0.34 ± 0.08	0.28 ± 0.07	0.34 ± 0.03	0.39 ± 0.13
Phosphatidylcholine	11.74 ± 1.31 (6)	12.49 ± 1.18	11.48 ± 1.00	10.46 ± 0.24
Phosphatidylethanolamine	10.41 ± 1.00	9.61 ± 0.55	8.99 ± 1.08	9.66 ± 1.27
Phosphatidylinositol	1.23 ± 0.48	1.07 ± 0.43	1.14 ± 0.06	1.32 ± 0.13
Phosphatidylserine	1.80 ± 0.31	1.55 ± 0.25	1.75 ± 0.03	1.89 ± 0.28
Diacylglycerol[b]	6.54 ± 0.48	7.40 ± 1.24	7.30 ± 1.28	5.94 ± 1.04

[a]Hamster hearts were perfused with Krebs-Henseleit buffer containing 1.0 mM glycerol for 60 min. The buffer was saturated with either 95% O_2 (control) or 95% N_2 (hypoxic), and contained either 0 or 0.5 mg/mL lidocaine. After perfusion, the lipid fractions were separated by thin-layer chromatography, and the amount of lipid in each fraction was determined as described in the Materials and Methods section. The results are expressed as means ± SD for n sets of experiments, unless indicated otherwise in parentheses.
[b]Diacylglycerol content is expressed as μmol glycerol/g wet weight.

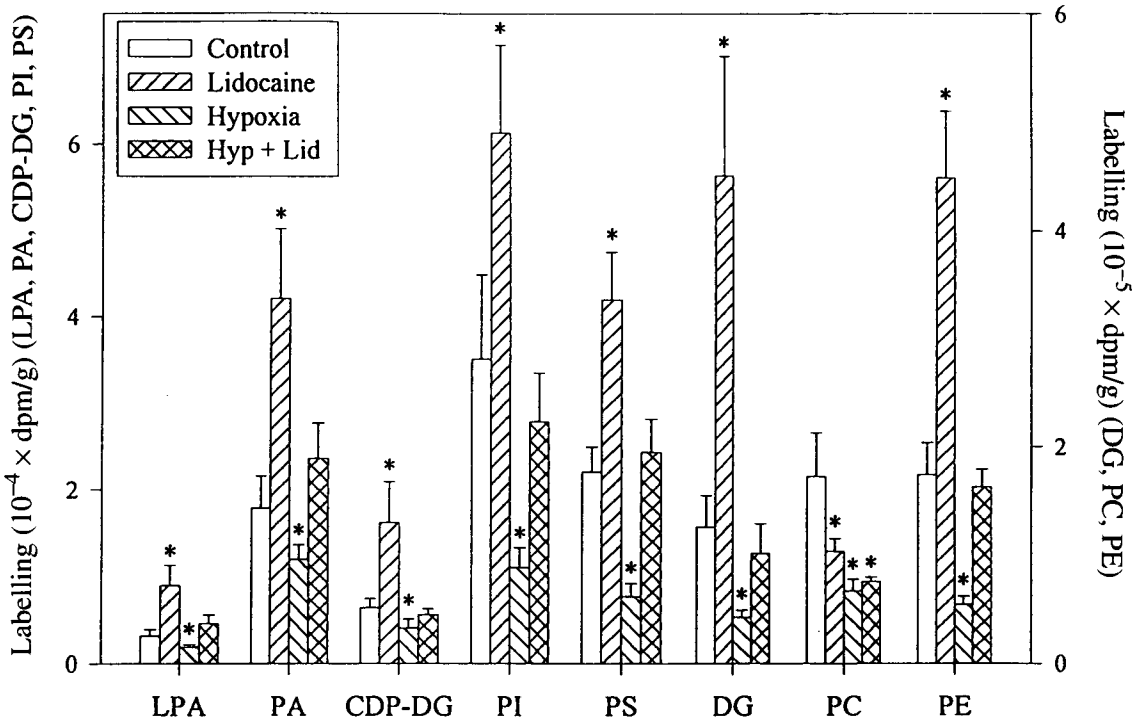


FIG. 2. The effects of lidocaine and hypoxia on the labeling of phospholipids and neutral lipids in the hamster heart. Hamster hearts were perfused as described in Figure 1. After perfusion, lipid fractions were separated by thin-layer chromatography (TLC) as described in the Materials and Methods section, and the radioactivity in each fraction was determined. The results are expressed as means ± SD. The numbers of experiments were as in Figure 1. Left axis corresponds to results for lysophosphatidic acid (LPA), phosphatidic acid (PA), CDP-diacylglycerol (CDP-DG), phosphatidylinositol (PI), and phosphatidylserine (PS). Right axis corresponds to results for diacylglycerol (DG), phosphatidylcholine (PC), and phosphatidylethanolamine (PE). *$P < 0.05$ compared with control.

hamster heart was not changed by hypoxic perfusion or lidocaine treatment, or by lidocaine under hypoxia.

Effects of lidocaine and hypoxia on phospholipid biosynthetic enzymes. Changes in the labeling of the cardiac phospholipids might arise from changes in activities of the enzymes responsible for their biosynthesis. Hence, the activities of the phospholipid biosynthetic enzymes in the hamster heart were determined subsequent to perfusion under hypoxia or with lidocaine. Specifically, hearts were perfused under the defined conditions for 60 min with Krebs-Henseleit buffer containing 1.0 mM glycerol. After perfusion, subcellular fractions were prepared and enzyme activities were determined as described in the Materials and Methods section. The activity of phosphatidic acid phosphatase was elevated (26%) in the microsomal fraction after lidocaine perfusion (Fig. 3). This effect was abolished when the microsomal fraction was incubated with 4.0 mM *N*-ethylmaleimide (NEM) prior to the assay of enzyme activity (data not shown). The increase in microsomal enzyme activity was accompanied by a 30% decrease in cytosolic enzyme activity. Perfusion under hypoxia decreased the activity of this enzyme in both microsomal and cytosolic fractions. The presence of lidocaine during hypoxic perfusion did not result in any beneficial effect on the phosphatidic acid phosphatase activity (Fig. 3).

The activity of CTP:phosphatidic acid cytidylyltransferase (for the formation of CDP-diacylglycerol) was not affected by lidocaine under normoxic conditions (Fig. 3). However, its activity was increased (52%) under hypoxia, and the presence of lidocaine in the hypoxic buffer caused a further increase (170%) above the control activity.

The activities of the other phospholipid biosynthetic enzymes examined were not significantly affected by various experimental treatments (Fig. 3). Although the labeling of phosphatidylcholine was decreased by lidocaine, the activity

TABLE 2
The Effect of Lidocaine on Nucleotide Levels in Normoxic and Hypoxic Hamster Hearts[a]

	Nucleotide level (% of control)		
Nucleotide	Lidocaine	Hypoxia	Hypoxia + lidocaine
ATP	112 ± 11	23 ± 18[b]	29 ± 10[b]
CTP	106 ± 10	48 ± 10[b]	35 ± 8[b]
CDPcholine	103 ± 14	105 ± 9	96 ± 11

[a]Hamster hearts were perfused for 60 min with Krebs-Henseleit buffer with or without 0.5 mg/mL lidocaine under normoxic or hypoxic conditions. After perfusion, ATP, CTP, and CDPcholine levels in the heart were determined as described in the Materials and Methods section. Results are expressed as percentage of control and values represent means ± SD for three sets of experiments. Control values were: ATP, 2.54 ± 0.16 μmol/g heart; CTP, 11.49 ± 2.24 nmol/g heart (5); CDPcholine, 94 ± 6 nmol/g heart.
[b]$P < 0.05$ compared with controls.

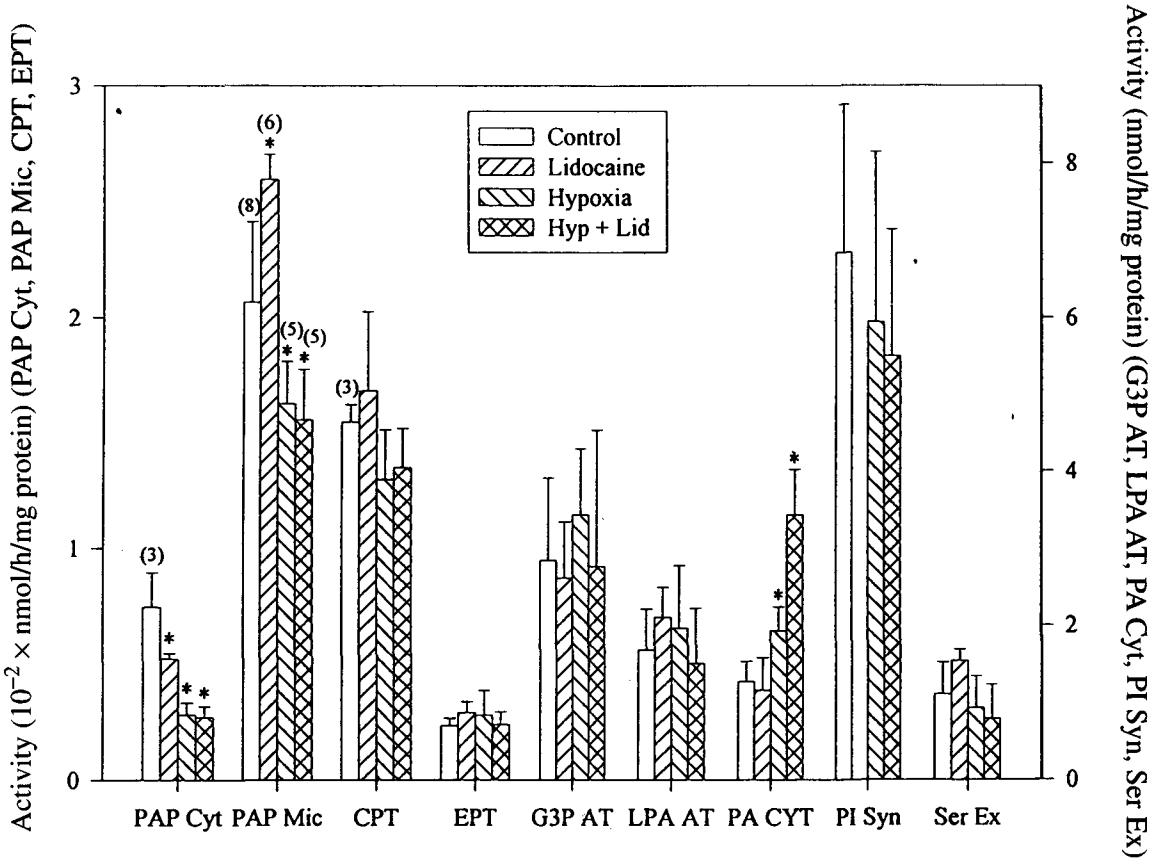


FIG. 3. The effects of lidocaine and hypoxia on the activities of lipid biosynthetic enzymes in the hamster heart. Hamster hearts were perfused for 60 min with Krebs-Henseleit buffer containing 1.0 mM glycerol. The buffer was saturated with either 95% O_2 (control) or 95% N_2 (hypoxic), and contained either 0 or 0.5 mg/mL lidocaine. After perfusion, subcellular fractions were prepared and enzyme activities were assayed as described in the Materials and Methods section. Results are expressed as means ± SD. The number of experiments was as follows, unless otherwise indicated in parentheses: control, $n = 6$; lidocaine, hypoxia, and hypoxia + lidocaine, $n = 3$. Left axis corresponds to results for phosphatidic acid phosphatase in the cytosolic fraction (PAP Cyt); and phosphatidic acid phosphatase (PAP Mic), CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT), and CDPethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (EPT) in the microsomal fraction. Right axis corresponds to results for acyl-CoA:glycerol-3-phosphate acyltransferase (G3P AT), acyl-CoA:lysophosphatidic acid acyltransferase (LPA AT), CTP:phosphatidic acid cytidylyltransferase (PA CYT), phosphatidylinositol synthase (PI Syn) (activity not determined for lidocaine perfusion), and serine base exchange enzyme (Ser Ex) in the microsomal fraction of the heart. *$P < 0.05$ compared with control.

CDPcholine:1,2-diacylglycerol cholinephosphotransferase, which catalyzes the incorporation of the diacylglycerol into phosphatidylcholine, was not changed following perfusion with lidocaine (Fig. 3). However, the direct addition of lidocaine to the assay mixture caused a decrease in the cholinephosphotransferase activity in both normoxic and hypoxic hearts, regardless of whether lidocaine was present in the perfusate during perfusion (Table 3). The direct addition of lidocaine to enzyme assays did not cause any significant change in the activities of phosphatidic acid phosphatase or CTP:phosphatidic acid cytidylyltransferase (data not shown).

TABLE 3
The Effect of Lidocaine on CDPcholine:Diacylglycerol Cholinephosphotransferase Activities[a]

Microsomal fraction	CDPcholine:diacylglycerol cholinephosphotransferase activity (nmol/h/mg protein)
Unperfused hearts	164 ± 20
Perfused control	159 ± 26
Perfused control + 0.5 mg/mL lidocaine	90 ± 14[b]
Perfused with lidocaine	165 ± 36
Perfused with lidocaine + 0.5 mg/mL lidocaine	94 ± 33[b]
Hypoxic perfusion	138 ± 26
Hypoxic perfusion + 0.5 mg/mL lidocaine	88 ± 23[b]

[a]Hamster hearts were perfused for 60 min with Krebs-Henseleit buffer containing 0 or 0.5 mg/mL lidocaine under normoxic or hypoxic conditions. After perfusion, microsomal fractions were isolated by differential centrifugation. Cholinephosphotransferase activities in the microsomal fractions were determined in the absence or presence of 0.5 mg/mL lidocaine. Results are expressed as means ± SD for four separate experiments.
[b]$P < 0.05$ compared with controls.

DISCUSSION

The objective of the present study was to investigate the effect of lidocaine on phospholipid biosynthesis under hypoxic conditions. We have shown earlier that a higher than therapeutic dose of lidocaine (for the treatment of cardiac arrhythmia; initial dose of 200 mg administered over 15 min) is required for a general stimulation of phospholipid biosynthesis

in the hamster heart (7). In this study, hypoxia was found to cause a general decrease in phospholipid biosynthesis. We have also shown that a high level of lidocaine has the ability to partially restore the decrease in phospholipid biosynthesis produced by hypoxia.

Because both ATP and CTP are required for the formation of phospholipids, changes in their levels during hypoxia would affect the labeling of phospholipids. The reduction in the levels of high energy nucleotides during hypoxia has been documented (5,29) and was confirmed in this study. The reduction of the high energy nucleotides would be coupled with increases in the monophosphonucleotides and diphosphonucleotides. The higher levels of these nucleotide species might further reduce the rate of phospholipid biosynthesis either through product inhibition or enhancement of the backward reaction. Interestingly, lidocaine has the ability to partially restore phospholipid synthesis during hypoxia without any significant effect on the reduced ATP and CTP levels.

CTP:phosphatidic acid cytidylyltransferase is the rate-determining enzyme for the synthesis of CDP-diacylglycerol, a precursor of phosphatidylinositol (30,31). The enhancement of its activity during hypoxia may be regarded as a compensatory mechanism to maintain the proper level of CDP-diacylglycerol at low CTP concentrations. This is not surprising because a similar compensatory increase in the activity of CTP:phosphocholine cytidylyltransferase has been previously observed (5). Alternatively, the ability of lidocaine to stimulate the enzyme activity only under hypoxic conditions is intriguing, and further studies are required to characterize the mechanisms for the observed increases in the enzyme activity.

Phosphatidic acid phosphatase catalyzes the conversion of phosphatidic acid to diacylglycerol and is regarded as a key enzyme in regulating the supply of diacylglycerol for the synthesis of phosphatidylcholine, phosphatidylethanolamine and triacylglycerol (for review see Ref. 32). There are two distinct forms of this enzyme. One form is located in the plasma membrane and is involved in signal transduction, while the other is found in the cytosolic and microsomal fractions and is involved in glycerolipid synthesis when bound to membranes (33,34). The latter form is characterized by its sensitivity to inhibition by the thio-reactive agent NEM, whereas the former is insensitive to NEM. The enhancement of the microsomal (NEM-sensitive) phosphatidic acid phosphatase activity by lidocaine may provide an explanation for the observed increase in the labeling of diacylglycerol. The increase in the microsomal enzyme activity might arise from the translocation of the enzyme from the soluble to the membrane compartment during lidocaine perfusion. Intriguingly, hypoxic perfusion caused a decrease in the enzyme activity in both cytosolic and microsomal fractions which was not reversible by lidocaine. Therefore, the restoration of diacylglycerol labeling by lidocaine under hypoxia might be caused by the enhanced synthesis of its metabolic precursors. Although no changes in the activities of acyl-CoA:glycerol-3-phosphate and acyl-CoA lysophosphatidic acid acyltransferases were detected when these enzymes were assayed after perfusion, their activities were stimulated by the direct addition of lidocaine to the assay mixtures (7). We postulate that the direct action of lidocaine on these enzymes might not be detectable when assayed after perfusion of the heart in the presence of lidocaine due to extensive dilution of the drug during the homogenization process.

The synthesis of phosphatidylcholine was distinct from the other phospholipids because it was inhibited by lidocaine. Our results indicate that CDPcholine:1,2-diacylglycerol cholinephosphotransferase plays a major role in the synthesis of phosphatidylcholine. During lidocaine perfusion, the labeling of phosphatidylcholine decreased under normoxia and remained depressed under hypoxia despite the lidocaine-induced increase or restoration, respectively, in the labeling of diacylglycerol. In addition, the CDPcholine pool was not changed by lidocaine or hypoxic perfusion. Thus, the *in situ* activity of the cholinephosphotransferase appears to be inhibited by lidocaine under both normoxic and hypoxic treatment. This notion is supported by the fact that the enzyme activity was shown to be inhibited by the direct addition of lidocaine to the assay mixtures. In contrast to phosphatidylcholine, the labeling of phosphatidylethanolamine correlated proportionately with the labeling of diacylglycerol under all perfusion conditions. Although diacylglycerol is the common precursor of phosphatidylcholine and phosphatidylethanolamine, it is clear that there are distinct and independent controls for the biosynthesis of these two phospholipids in the hamster heart.

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Effectiveness of Resistant Starch, Compared to Guar Gum, in Depressing Plasma Cholesterol and Enhancing Fecal Steroid Excretion

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ABSTRACT: Amylase-resistant starch (RS) represents a substrate that can be administered in substantial amounts in the diet, in contrast to gel-forming polysaccharides, such as guar gum (GG). The aim of this work was thus to compare the effects of GG and RS on cholesterol metabolism in rats adapted to 0.4% cholesterol diets, using dietary GG or RS levels (8 or 20%, respectively) that led to a similar development of fermentations, as assessed by the degree of enlargement of the cecum. The RS diet elicited a marked rise in the cecal pool of short-chain fatty acids, especially acetic and butyric acid, whereas the GG diet favored high-propionic acid fermentations. Both polysaccharides markedly altered the cholesterol excretion, from 50% of ingested cholesterol in controls, up to about 70% in rats adapted to the RS or GG diets. With these diets, the fecal excretion of bile acids was enhanced (67 and 144% with the RS and GG diets, respectively). RS and GG diets were effective in lowering plasma cholesterol (about −40%) and triglycerides (−36%). There was practically no effect of the diets on cholesterol in d > 1.040 lipoproteins (high density lipoproteins), whereas RS (and to a larger extent, GG) were very effective to depress cholesterol in d < 1.040 lipoproteins (especially in triglyceride-rich lipoproteins). Fermentable polysaccharides counteracted the accumulation of cholesterol in the liver, especially cholesterol esters. In parallel, liver acyl CoA:cholesterol acyltransferase was depressed in rats fed the RS or GG diets, whereas only the GG diet counteracted the downregulation of 3-hydroxy-3-methylglutaryl–CoA by cholesterol. These data suggest that RS may be practically as effective as a gel-forming gum, such as GG, on steroid excretion and on cholesterol metabolism.

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There has been a great deal of interest in the cholesterol-lowering properties exhibited by some dietary fibers. Most of these hypocholesterolemic fibers are viscous materials that are fermentable within the large intestine. Guar gum (GG; a gel-forming galactomannan polysaccharide) has been extensively investigated in this respect, and the hypocholesterolemic effect of this gum is apparently connected to: (i) its ability to elevate the viscosity in the small intestine, thus depressing cholesterol absorption (1,2) and the subsequent delivery of cholesterol to the liver *via* chylomicron remnant; (ii) its bile acid-binding capacity, which increases the catabolism of cholesterol through conversion to bile acids in the liver (3–5). Soluble fibers are generally broken down by the large intestine microflora, and the resulting production of short-chain fatty acids (SCFA) may be involved in the metabolic effects of fibers (6), besides modification in the secretion of gastrointestinal hormones, insulin, and glucagon (7,8). These last effects are certainly prevailing with fermentable carbohydrates which are devoid of ion-exchange or viscous properties, such as oligosaccharides or resistant starches (RS) (9–11), although it has been proposed that the helical structures in starch could act as binding sites for bile salts (12). In this view, it has been reported that, in the absence of fermentations in the large intestine, a high rate of bile acid excretion is not always sufficient to elicit a plasma cholesterol-lowering effect (13,14).

In this work, it was thus decided to compare the effects of GG and an RS (crude potato starch) on cholesterol metabolism by using levels in the diet leading to a similar development of fermentations, as assessed by the degree of enlargement of the cecum. The results indicate that, when given at a relatively low level in the diet, a gel-forming compound, such as GG, is effective on cholesterol metabolism, probably because of its capacity to influence most of the potential sites controlling this metabolism. A nonviscous fermentable polysaccharide, such as RS, is also effective to alter the digestive balance of neutral and acidic steroids, and could be tolerated at a higher dietary level than GG.

MATERIALS AND METHODS

Animals and diets. Three groups of eight male Wistar rats (IFA-CREDO, l'Arbresle, France) weighing 160 g, were housed two per cage (wire-bottomed to limit coprophagy) and

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Abbreviations: ACAT, acyl CoA:cholesterol acyltransferase; GG, guar gum; HDL, high density lipoprotein; HMGR, 3-hydroxy-3-methylglutaryl–CoA reductase; LDL, low density lipoprotein; RS, resistant starch; SCFA, short-chain fatty acid; TGRLP, triglyceride-rich lipoprotein.

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fed a semipurified diet that contained the following (by dry weight): 14% casein (L. François, Saint-Maur, France), 14% peanut oil (CIO, Genay, France), 0.4% cholesterol (Sigma, St. Louis, MO), 6% mineral mixture (UAR, Villemoisson/Orge, France), and 1% vitamin mixture (UAR). In the control diet, the carbohydrate supply was 64.6% wheat starch (L. François), whereas in the experimental groups, a part of wheat starch was replaced by 20% crude potato starch (L. François) or by 8% GG (Sigma). The rats were housed in a temperature-controlled room (22°C) with the dark period from 2000 to 0800 h for 21 d. The daily food consumption and body weight were recorded, and the feces were collected and weighed over three consecutive days (to study bile acid excretion and neutral sterol balance) at the end of the adaptation period (starting at day 18). Animals were handled according to the recommendations of the Institutional Ethic Commitee (Clermont-Ferrand University, Clermont-Ferrand, France).

Sampling procedures. Rats were sampled at the end of dark period (between 0800 and 0900 h), when cecal fermentations were still very active. They were anesthesized (sodium pentobarbital, 40 mg/kg) and maintained on a warming plate at 37°C. An abdominal incision was made and blood was withdrawn from the cecal vein and the abdominal aorta. The blood was placed in microfuge tubes containing heparin and centrifuged at 10,000 × *g* for 2 min. Plasma samples were kept at 4°C for lipid and lipoprotein analysis.

A portion of liver was freezed-clamped and stored at −80°C for the measurement of lipid contents. Hepatic microsomes were prepared as described previously (14) and stored at −80°C until measurement of 3-hydroxy-3-methylglutaryl–CoA reductase (HMGR) and acyl CoA:cholesterol acyltransferase (ACAT) activities. The protein content of the microsomes was determined using the Pierce BCA reagent kit (Interchim, Montluçon, France).

At the same time, the cecum was removed and weighed. Duplicate samples were collected into 2-mL microfuge tubes that were immediately frozen and stored at −20°C. Before SCFA analysis, supernatants were obtained by centrifuging one of these two microtubes at 20,000 × *g* for 10 min at 4°C.

Analytical procedures. SCFA were measured on aliquots of cecal supernatants by gas–liquid chromatography as described previously (15). Bile acids and neutral sterols were extracted from feces by dispersion using a Polytron homogeneizer (Luzern, Switzerland) in 10 vol of ethanolic KOH (0.5 M); after an additional extraction step, the dispersed samples were warmed at 80°C for one hour and then centrifuged at 8,000 × *g* for 5 min. Bile acids concentration was measured on this extract using the reaction catalyzed by 3 α-hydroxysteroid dehydrogenase (EC 1.1.1.50; Sigma) (16); cholesterol concentration was enzymatically determined on this same extract using a kit purchased from BioMérieux (Charbonnières-les-Bains, France). Liver lipids were extracted with chloroform/methanol (2:1, vol/vol) according to the method described by Folch *et al.* (17). Triglycerides (Biotrol, Paris, France), total cholesterol, and free cholesterol (Boehringer, Meylan, France) were determined by enzymatic procedures as described by Mazur *et al.* (18). Hepatic cholesteryl ester concentrations were calculated by difference between total and free cholesterol.

Plasma lipoproteins were separated by density gradient ultracentrifugation (19), using pooled samples. After centrifugation in a TST 41.14 swinging-bucket rotor at 100,000 × *g* for 24 h at 18°C, the gradient was divided into 24 × 500 µL fractions and kept at 4°C for lipid analysis. Due to the low level of low density lipoprotein (LDL) and the relative overlapping of high density lipoprotein (HDL)1 and HDL2 fractions in rat plasma, it was decided to present data on the $d < 1.040$ fraction [chiefly triglyceride-rich lipoprotein (TGRLP), with a minor contribution of LDL] and on the $d > 1.040$ fraction (essentially HDL), as previously effected (20).

Triglycerides (Biotrol), cholesterol, and phospholipids (BioMérieux) were determined in plasma and lipoprotein fractions by enzymatic procedures. The protein concentration of isolated lipoproteins was determined using the Pierce BCA reagent kit.

The activity of liver microsomal HMGR was measured as described by Wilce and Kroone (21): labeled mevalonolactone was separated from unreacted HMG–CoA by columm chromatography, using AG1-X8 resin (200-400 mesh; Biorad, Paris, France). The activity was expressd in pmol of [^{14}C]HMG–CoA transformed in [^{14}C]mevalonolactone per minute per milligram of microsomal protein. ACAT activity was measured on liver microsomes using the method described by Erickson *et al.* (22). Endogenous microsomal cholesterol was esterified in presence of [^{14}C]oleyl-CoA, and the resulting cholesteryl-[^{14}C]oleate was isolated after extraction by chloroform/methanol (2:1,vol/vol) followed by thin-layer chromatography. ACAT activity was expressed in nmoles of oleyl-CoA incorporated in cholesteryl oleate per minute per milligram of microsomal protein.

Statistical analysis. Values are given as the means ± SEM and, where appropriate, significance of the difference between mean values was determined by analysis of variance coupled with the Student-Newman-Keuls multirange test. Values of $P < 0.05$ were considered significant.

RESULTS

Effects of diets on animals and their digestive tract. The different diet conditions had no influence on the body weight gain (Table 1), but the daily food intake was slightly higher in rats fed the RS diet than in those fed the control or the GG diets (25.9 vs. 24.0 g/d, respectively). In rats fed the RS and GG diets, the caecum was markedly heavier (+130%) than in control rats. The cecal pH was 7.22 in rats fed the control diet; it was more acidic (6.21) in rats adapted to the RS diet, as well as in those adapted to the GG diet (5.91). RS diet led to a rise in the total SCFA concentration, compared to controls (from 82 to 153 mM), as well as in the total SCFA pool (from 149 to 674 µmoles). This rise was lesser in rats fed the GG diet (total SCFA 118 mM, corresponding to a cecal SCFA pool of 479 µmoles). There was a striking difference between the

TABLE 1
Effects of Diets on Animals and Their Digestive Tract[a]

		Cecum					
Diet	Body weight (g)	Total weight (g)	Content pH	Acetate (mM)	Propionate (mM)	Butyrate (mM)	Total SCFA (μmoles)
Control	250 ± 5	2.20 ± 0.20^b	7.22 ± 0.14^b	56 ± 6^b	18 ± 2^b	8 ± 1^b	149 ± 15^b
Resistant starch (20%)	256 ± 10	5.19 ± 0.58^c	6.21 ± 0.15^c	87 ± 7^c	27 ± 7^c	39 ± 5^c	674 ± 35^d
Guar gum (8%)	245 ± 4	5.08 ± 0.29^c	5.91 ± 0.12^c	58 ± 6^b	50 ± 6^d	10 ± 2^b	479 ± 26^c

[a]Values are means ± SEM for eight rats. Values with different superscript letters are significantly different at $P < 0.05$. SCFA, short-chain fatty acids.

TABLE 2
Effects of Diets on the Excretion of Bile Acids and the Digestive Balance of Cholesterol[a]

	Bile acids		Cholesterol		
Diet	Cecal pool (μmoles)	Fecal excretion (μmoles/d)	Daily intake (μmoles/d)	Fecal excretion (μmoles/d)	Excreted (% ingested)
Control	9.6 ± 0.6^b	18 ± 2^b	216 ± 12	109 ± 8^b	51
Resistant starch (20%)	14.9 ± 0.5^c	30 ± 5^c	243 ± 14	170 ± 10^c	70
Guar gum (8%)	20.1 ± 0.6^d	44 ± 8^c	225 ± 18	166 ± 17^c	74

[a]Values are means ± SEM for eight rats. Values with different superscript letters are significantly different at $P < 0.05$.

molar SCFA ratio in the cecum: RS diet led to high acetic/butyric acid fermentations, whereas GG diet rather promoted high propionic acid (42% of total SCFA) fermentations.

Effects of diets on the the fecal excretion of neutral and acidic steroids. As shown in Table 2, the daily cholesterol intake in rats fed the control or GG diet was about 220 μmoles/d, except in rats adapted to the RS diet (243 μmoles/d) due to the greater food intake in this diet group. The cholesterol fecal excretion in controls (109 μmoles/d) corresponded to 50% of the daily food supply. This excretion was markedly elevated in rats adapted to the fiber diet (55%), and it corresponded to 70% (RS diet) or 74% (GG diet) of the daily cholesterol intake. Both RS and GG diets elicited a significant rise in the cecal bile acids pool (54 and 110%, respectively), whereas the cecal bile acids concentration was rather depressed, especially in rats adapted to the RS diet. The daily fecal excretion of bile acids was 67% greater than in controls in rats fed the RS diet and 144% greater in those fed the GG diet.

Effects of RS or GG on plasma lipids and lipoprotein profile, and on liver lipids. Rats fed the RS or GG diets had a significantly lower plasma cholesterol and triglycerides concentration than control rat (Table 3), but the cholesterol-lowering effect was more pronounced in rats adapted to the GG diet. Analysis of the plasma lipoprotein profile (Fig. 1) shows that cholesterol in the $d < 1.040$ kg/L fractions, in rats adapted to the control cholesterol diet, accounted for 68% of total lipoprotein cholesterol. There was a drastic reduction of cholesterol in this fraction (particularly in TGRLP, data not shown) in rats fed the RS or GG diets (GG being the most potent). In contrast, there was no significant change in the $d > 1.040$ cholesterol (essentially HDL) in rats adapted to the RS or GG diets. The triglyceride concentration in the TGRLP fraction was also significantly depressed by RS and GG (−22 and −28%, respectively).

Both fermentable polysaccharides displayed a significant effect on liver pool of cholesterol and triglycerides, but the GG diet appeared more potent in this respect than the RS diet (Table 3). There was no significant effect of the diets on the liver-free cholesterol, and the lowering of liver cholesterol essentially affected the esterified fraction; as a result, the percentage of esterified cholesterol declined from 70% to about 50%.

Effects of RS or GG on the liver HMGR and ACAT activity. As shown in Figure 2, the microsomal HMGR activity was practically suppressed (18 pmoles · min^{-1} · milligram protein^{-1}) in rats fed the control 0.4% cholesterol diet, and it was not significantly modified in those adapted to the RS diet. In contrast, the HMGR activity was markedly higher (71

TABLE 3
Effects of Diets on the Plasma and Liver Concentrations of Cholesterol and Triglycerides[a]

	Plasma		Liver		
Diet	Triglycerides (mM)	Cholesterol (mM)	Triglycerides (mg/g)	Cholesterol (free) (mg/g)	Cholesterol (esters) (mg/g)
Control	1.57 ± 0.27^b	2.67 ± 0.18^b	21.50 ± 2.60^b	2.49 ± 0.25^b	5.81 ± 0.38^b
Resistant starch (20%)	1.01 ± 0.21^c	1.77 ± 0.12^c	16.44 ± 1.56^c	2.62 ± 0.37^b	2.57 ± 0.15^c
Guar gum (8%)	1.00 ± 0.19^c	1.56 ± 0.10^c	14.06 ± 1.28^c	1.93 ± 0.16^c	2.03 ± 0.24^d

[a]Values are means ± SEM for eight rats. Values with different superscript letters are significantly different at $P < 0.05$.

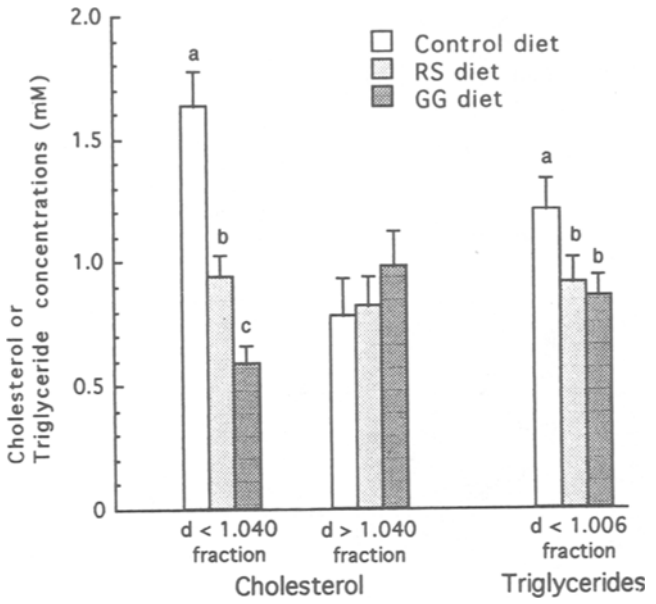


FIG. 1. Histograms showing the repartition of cholesterol and triglycerides in the various plasma lipoprotein fractions in rats adapted to the control, resistant starch (RS), or guar gum (GG) diets. Each value is a mean ± SEM of a triplicate analysis of a pool of eight plasmas. The fractions with a density lower than 1.040 kg/L corresponded chiefly to triglyceride-rich lipoproteins, with a minor contribution of low density lipoproteins. The fractions with a density higher than 1.040 kg/L corresponded essentially to high density lipoproteins. Values not sharing a common letter are significantly different ($P < 0.05$).

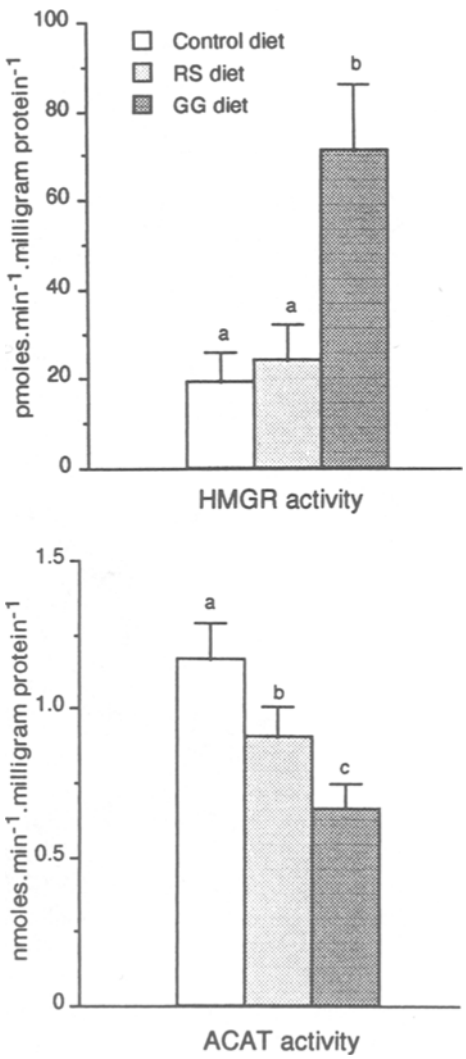


FIG. 2. Histograms showing the effects of diets on the hepatic activity of 3-hydroxy-3-methylglutaryl–CoA reductase (HMGR) and acyl CoA:cholesterol acyltransferase (ACAT). Each value is a mean ± SEM for eight rats. Values not sharing a common letter are significantly different ($P < 0.05$). See Figure 1 for abbreviations.

pmoles · min^{-1} · milligram $protein^{-1}$) in rats adapted to the GG diet. The basal ACAT activity present at a relatively high level (1.15 nmoles · min^{-1} · milligram $protein^{-1}$) in rats fed the fiber-free/cholesterol diet; ACAT activity was slightly lowered in rats fed the RS diet, but it was strongly depressed (about –45%) in those adapted to the GG diet.

DISCUSSION

The RS or GG diets led to a considerable increase in cecal weight, a general feature of fermentable carbohydrates in rats (23). However, the availability of polysaccharide for microbial degradation could be somewhat higher with the RS than with GG diet (although there was no difference in the cecal weight), as the cecal SCFA pool was 40% higher in rats adapted to the RS diet. RS promoted high-SCFA fermentations, characterized by a large proportion of butyric acid, whereas GG favored high-propionic acid fermentations. As a result, the cecal pool of propionate was 70% higher in rats adapted to GG than to the RS diet. Fermentable carbohydrates may alter the energetic fuel available for liver metabolism, corresponding to a partial substitution of glucose by SCFA, especially with RS (8). Furthermore, the time course of glucose absorption could be prolonged by the presence of GG but also when a significant part of starch is resistant to pancreatic hydrolysis. Only a small percentage of absorbed glucose is removed and utilized by the liver (8), whereas SCFA are essentially taken up by the liver, especially propionic and butyric acid (6). This is accompanied by a lowering of the basal insulinaemia and of the daily fluctuations of plasma insulin (8).

There was an unequivocal hypocholesterolemic effect of GG or RS in the present study performed on rats fed 0.4% cholesterol diets. The cholesterol-lowering effect of RS was less apparent with low fat/no cholesterol diets (8,14). Accordingly, soluble fibers, such as GG or pectin, more effectively depressed plasma cholesterol with cholesterol diets (1,2,23). With the present cholesterol diets, the first consequence of polysaccharides intake seems to be a reduced effectiveness of cholesterol absorption. With the GG diet, the high viscosity of the intestinal content may slow down triglycerides digestion, shifting intestinal fatty acids absorption toward more distal sites (24). In contrast to glycerides, only a part of dietary cholesterol is absorbed, especially when it is present in

relatively high quantities in the diet. It is noteworthy that RS appeared as effective as GG to enhance the fecal excretion of cholesterol.

More than 60% of crude potato starch escapes to pancreatic hydrolysis (25), and thus its helical structure could be available as binding sites for steroids (12), but it is still unclear whether cholesterol is as effectively trapped as bile salts in these structures. Accumulation of bile acids in the cecum is probably a reflection of impaired reabsorption in the small intestine, especially the ileum. Thus, it appears that GG is very effective in enhancing the appearance of bile acids in the large bowel, but RS is also effective in this respect. Gel-forming properties of GG have been frequently invoked; some data indicates that GG actually binds bile acids, but not phospholipids, suggesting that its trapping properties are relatively specific (26). It must be kept in mind that, due to the hydrolysis and absorption of most nutrients, the digestive content in the distal jejunum and the ileum should be strongly enriched into nonavailable carbohydrates, together with endogenous materials. This could explain why compounds with low (or devoid of) bile acids binding capacities are liable to affect bile acids reabsorption. It has also been suggested that, proportionately, bile acids sequestrants may have a greater effect in the colon than in the upper portion of the gastrointestinal tract (27).

In the cecum, bile acids are extensively deconjugated and converted to secondary bile acids by bacterial 7α-dehydroxylation. A passive absorption of bile acids takes place in the large intestine, but the fact that their fecal excretion is enhanced indicated that absorption in the large intestine (which may be enhanced with soluble fibers, Ref. 23) fails to compensate for a reduce absorption in the small intestine. Secondary bile acid absorption is likely to be depressed, because of (i) inhibition of 7α-dehydroxylase activity at acidic pH (28), (ii) depressed bile acid solubility due to acidic conditions, especially as insoluble calcium salts (29). Furthermore, bacterial proliferation is itself a factor of bile acids insolubilization because bile acid binding possibilities by colonic microorganisms have been reported (30,31).

The present data suggests that the digestive absorption of cholesterol could be lowered from 50% in control rats down to 26–30% in rats fed the RS or GG diets, as reported for GG in guinea pig (2). Using retrograded starch (RS3 type), Verbeek *et al.* (32) actually found a stimulation of fecal bile acid excretion, but a lowered excretion of neutral steroids. It must be kept in mind that this balance is not a direct reflection of intestinal absorption as it is also affected by changes in the endogenous secretion of cholesterol. Nevertheless, the drastic lowering of cholesterol in the $d < 1.040$ fraction (apoprotein B-rich lipoproteins) of rats adapted to the RS or the GG diets (triglyceride-lowering was less effective) is consistent with a reduced rate of absorption from the digestive tract, although other factors (involved in the control of the peripheral clearance of TGRLP) also have an influence (33). Addition of soluble fibers to the diet has been shown to decrease plasma LDL cholesterol concentrations in species in which cholesterol was transported by LDL (34–36). A large part of the plasma cholesterol in rats is associated with HDL. With cholesterol-free diets, it has been observed that soluble fibers exert a cholesterol-lowering effect in all lipoprotein fractions, including HDL (4,10,14). The presence of a large part of plasma cholesterol in TGRLP in rats fed cholesterol could be due to a reduced rate of chylomicrons clearance, together with a high rate of cholesterol appearance (from digestive origin). Furthermore, it has been reported that cholesterol-enriched diets are liable to stimulate triglyceride synthesis and to depress the oxidation of fatty acids in the liver (37). It is noteworthy that fermentable carbohydrates, particularly GG, were very effective to depress cholesterol in the $d < 1.040$ fraction. This could result from an impaired intestinal absorption of cholesterol, altered concentrations of circulating apolipoproteins (4,38), or from an accelerated rate of lipoprotein removal by tissues (18,39,40). Furthermore, the increase in bile acid synthesis (as reflected by the rate of fecal bile acid excretion) could also represent a mechanism to explain the reduction of cholesterol in the TGRLP.

The liver is the major organ involved in cholesterol homeostasis, because it exhibits capacities to *de novo* synthesize, to oxidize, or to esterify cholesterol. Hepatic ACAT activity is generally elevated when the cholesterol delivery to the liver is enhanced (40,41). In the present study, RS and GG diets elicited a lowering of liver cholesterol, particularly the esterified fraction. This was accompanied by a noticeable decrease in ACAT activity, as reported elsewhere (2,40,41). A depressed esterification of cholesterol may correspond to a decrease in the amount of enzyme or to the influence of regulating intracellular factors. It could also be related to changes in the substrates pools (cholesterol and fatty acyl-CoA). Free cholesterol was slightly depressed in the liver of rats adapted to the GG diet, but not in those fed the RS diet. A decrease in the microsomal cholesterol concentration may account for a diversion of the intracellular pool of cholesterol from the ACAT sites to the cholesterol 7α-hydroxylase sites, which have been inducted with GG diets (3,4). This should reduce the pool of cholesterol esters destined to the core of the very LDL, hence an improved vascular catabolism of these particles (33). GG intake was more efficient than RS in lowering plasma cholesterol and the free-cholesterol fraction in the liver; accordingly, only GG counteracted the repression of HMGR caused by dietary cholesterol. With cholesterol-free diets, the loss of bile acids induced by RS feeding is generally sufficient to elicit an induction of liver HMGR (14). Bile acid elimination is certainly enhanced by fibers, and they have recently been described as upregulators of ACAT, with little effect on HMGR activity (42).

The question arises whether SCFA, particularly propionate, potentiate the consequences of an enhanced fecal steroid excretion. A putative role of this SCFA to mediate the cholesterol-lowering effect of fiber has been proposed (43), probably in relation with the inhibition of the metabolism of the major lipogenic precursors, such as acetate or lactate (8,44). A relatively low insulinemia has been observed in rats

fed RS, in parallel to a high rate of SCFA absorption while glucose absorption was lower than in fiber-free controls; taken together, these factors may account for a depressed lipid synthesis (9). The relationship between SCFA availability and cholesterol metabolism is certainly complex as, in the present study: (i) an RS diet which yields acetic/butyric acid fermentations was effectively hypocholesterolemic, (ii) a GG diet which yields high-propionic acid fermentations was even more effective, although there was an upregulation of HMGR.

In conclusion, the study indicates that both nonviscous (RS) or viscous (GG) nonavailable polysaccharides may exhibit a cholesterol-lowering effect in animals adapted to a cholesterol diet. The commited mechanisms are certainly complex and affect different processes—absorption in the small intestine, large bowel fermentations, hepatic and extrasplanchnic metabolism. Compounds like GG are difficult to administer in relatively large quantities, and it appears that, in this respect, RS represent an interesting alternative and/or complement for maximal effects on cholesterolemia.

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Influence of a Subinhibitory Dose of Antifungal Fatty Acids from *Sporothrix flocculosa* on Cellular Lipid Composition in Fungi

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ABSTRACTS: Antifungal fatty acids produced by the biocontrol fungus *Sporothrix flocculosa* were studied on the basis of their effect on growth and cellular lipid composition of three fungi, *Cladosporium cucumerinum, Fusarium oxysporum,* and *S. flocculosa,* whose growth was decreased by 51, 33, and 5%, respectively, when exposed to 0.4 mg fatty acid per ml. The sensitivity to fatty acid antibiotics from *S. flocculosa* was related to a high degree of unsaturation of phospholipid fatty acids and a low proportion of sterols. The major responses of sensitive fungi to sublethal doses of antifungal fatty acids from liquid culture of *S. flocculosa* were: (i) a decrease in total lipid; (ii) an increase in the degree of fatty acid unsaturation (18:1 > 18:2 > 18:3); (iii) an increase in free fatty acids and phosphatidic acid and a decrease in total phospholipids; and (iv) an increase in sterol/phospholipid ratio. These modifications in lipid composition led to an increase in membrane fluidity in sensitive fungi, as demonstrated by assessment of fluoresence anisotropy using liposomes and 1,6-diphenyl-1,3,5-hexatriene probe. This alteration in the physical state of lipids appears to be responsible for the previously demonstrated alteration of membrane structure and function in fungi confronted to *S. flocculosa.*
Lipids 31, 1077–1082 (1996).

Sporothrix flocculosa Traquair, Shaw and Jarvis, is an antagonist fungus which has biocontrol potential against powdery mildew diseases (1–4). Microscopic and ultrastructural observations revealed that the antagonist induced a collapse of host conidia and mycelium without hyphal penetration (3,5). These results suggested that *S. flocculosa* killed its hosts by means of antibiosis (5,6). Chemical analysis of the culture filtrates confirmed this hypothesis and led to the isolation of three molecules with antimicrobial properties (7,8). They were identified as 4-methyl-7,11-heptadecadienoic acid, 9-heptadecenoic acid, and 6-methyl-9-heptadecenoic acid. When tested *in vitro,* these molecules exhibited a spectrum of activity against several fungi, but were not active against *S. flocculosa* itself (6,7). Additional work indicated that these metabolites induced the release of intracellular ions and proteins from treated fungi (6). Theses results combined with cytochemical observations (5) suggested that these antibiotics produced by *S. flocculosa* caused a disruption of the properties and function of the cytoplasmic membrane.

The antifungal properties of fatty acids have received considerable attention over the years (9–12). From previous work, it is known that unsaturated fatty acids can inhibit the activity of several membrane proteins, such as α-mannosyltransferase (13), chitin-synthase (14), valyinyl-tRNA synthetase (15), and (1,3)-β-glucan synthase (16). However, little is known about the mechanisms by which these fatty acids interfere with the activity of these proteins. The interaction between exogenous fatty acids and membrane lipids has been proposed as a key factor to explain the observed inhibitory effects (16), but Kabara *et al.* (11) suggested that the fluidity of the cell membrane can be disturbed maximally by lipophylic compounds of particular chain lengths or unsaturation.

In this study, we have attempted to elucidate the molecular basis underlining the mechanisms by which *S. flocculosa* antibiotics altered membrane permeability. Considering the importance of lipids in maintaining the physiological properties of cell membrane, it was hypothesized that the antimicrobial fatty acids interfered with lipid composition in the target fungi. In this context, the objectives of this study were twofold—to compare and to analyze the lipid composition of three fungi, *Cladosporium cucumerinum, Fusarium oxysporum,* and *S. flocculosa,* reported to be sensitive, moderately sensitive, and resistant, respectively, to the antibiotics; and to compare changes in lipid composition following an exposure to a subinhibitory level of antibiotics.

MATERIALS AND METHODS

Fungal material. Sporothrix flocculosa was maintained on yeast malt agar containing 3 g yeast extract/L, 3 g malt extract/L, 5 g peptone extract/L, 10 g dextrose/L, and 15 g bactoagar/L. *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) Jarvis and Shoemaker was obtained from P. Thibodeau (MAPAQ, Quebec, Canada) and *C. cucumerinum* Ellis and Arth from the Centre for the Land and Resources Research (Ottawa, Canada). FORL and *C. cucumerinum* were maintained on potato/dextrose agar.

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; FAME, fatty acid methyl ester; FORL, *Fusacium oxysporum* F. Sp. *radicis-lycopersici,* PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; S/P, sterol/phospholipid ratio; TLC, thin-layer chromatography.

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Extraction of antifungal fatty acids from liquid culture of S. flocculosa. Antibiotics were extracted as described previously (7,8). Briefly, the liquid medium was separated from the biomass through filter paper (Whatman No. 1) and the mycelium discarded. The filtrates were further extracted with chloroform. The organic fraction was dried under vacuum and the residue was redissolved in chloroform before analysis by thin-layer chromatography (TLC). Chromatograms were developed in ethyl acetate/hexane (1:4, vol/vol) and spots were viewed under ultraviolet radiations at 254 and 366 nm. To localize zones of antifungal activity, silica gel plates were seeded with a sporal suspension of *C. cucumerinum,* as described previously (17). In addition, both oleic and linoleic acids were bioassayed at concentrations of 1, 10, 100, and 1000 μg to serve as controls and to assess specificity of the antifungal fatty acids. The chloroform extracts from the filtrates were applied to a silica gel column and flash-chromatographed by elution with hexane, diethyl ether, and methanol. The active fractions were purified with a combination of preparative TLC and high-performance liquid chromatography with C_{18} reverse-phase columns (8 × 10 cm; Millipore-Waters Ltd., Mississauga, Ontario, Canada). Identification of the active fractions was performed by Fourier transform infrared spectra, nuclear magnetic resonance magnetic spectroscopy (^1H, ^{13}C, 2-D, and Hector) and gas chromatography/mass spectrometry.

Effect of antibiotics on fungal growth. Conidia from tested fungi were suspended in 100 mL of yeast malt peptone dextrose broth medium at 10^6 cells/mL. The three antifungal fatty acids, in a ratio 1:1:1 (by vol) were tested at a concentration (0.4 mg/mL) which reduced the dry weight growth of *C. cucumerinum* by approximately 50% (Table 1). This relatively high concentration was necessary to achieve the desired effect because of the low solubility of the fatty acids in a water-based medium. The antibiotics were added to the sterile medium as a methanol solution. Growth was quantified by dry weight measurement after four days. A comparable quantity of methanol was added to the culture media to serve as control.

Extraction of lipids. Fungal cells were collected by centrifugation, washed with water, and dried by lyophilization. Lyophyllized cells were suspended in 50 mL methanol and homogenized with a Polytron (Kinematica, Brinkman Instruments, Ontario, Canada) for one minute. Chloroform and water were then added to the suspension to give a ratio of 2:2:1 (by vol) chloroform/methanol/water. The suspensions were placed on a rotary shaker at 150 rpm in the dark for 12 h. The cells were collected by centrifugation (8000 × *g*) and the extraction procedure was repeated with a ratio of 4:2:1 (by vol) of methanol/chloroform/water. After 24 h the combined extracts were dried with anhydrous Na_2SO_4 and the organic phases were taken to dryness on a rotary evaporator and the residue immediately dissolved in chloroform/methanol (2:1, vol/vol) and concentrated under nitrogen gas. The oil residue constituted the total lipid extract. It was kept at 4°C until use.

Analysis of lipids. Polar lipids, mainly phospholipids, and neutral lipids were separated by precipitation in acetone as described by Kates (18). Individual phospholipids were separated by TLC on Silica Gel 60 (0.5-mm thick) (VWR Scientific, London, Ontario, Canada) using a solvent system of chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1; by vol). Phospholipid classes were located by exposing plates to iodine vapor. Individual phospholipids were identified by reference to standards (Sigma Chemicals, St. Louis, MO) run simultaneously. Using this procedure, phosphatidylinositol and phosphatidylserine were not completely separated. The silica gel bands corresponding to each compound were scraped from the plates and washed by neutral Bligh-Dyer solvent as described by Kates (18). Phospholipid classes were quantified by the phosphorus assay as described by Marinetti (19).

Neutral lipids were separated by TLC in hexane/diethyl ether/acetic acid (7.8:2:0.4, by vol). All neutral lipid classes were visualized on the plates with iodine vapor and identified by comparison of R_f values with authentic standards (Sigma).

Total fatty acids and sterols were obtained by alkaline hydrolysis (0.8 mL, 33% wt/vol, KOH in 8 ml EtOH, 2 h at 90°C). The nonsaponifiable fraction containing sterols was obtained by washing the hydrolysate three times with 15 mL hexane. The hydrolysate was then brought to pH 1–2 with 6 M HCl, and the saponifiable fraction containing the fatty acids was obtained by washing it three times with pentane. Fatty acids obtained in this manner were converted to their fatty acid methyl ester (FAME) derivatives using 14% BF_3/MeOH reagent (Sigma) at 90°C for 60 min under nitrogen. FAME were also prepared directly from phospholipids (obtained from TLC plates, as described previously) by transesterification using sodium methoxide in methanol. FAME were analyzed by gas–liquid chromatography (GLC) (model 5890 series II, Hewlett Packard, Mississauga, Ontario, Canada) on a 30–m capillary DB-225 column (J&W Scientific, Rancho Cordova, CA). The oven temperature program was initially 100°C, then raised at 20°C min^{-1} to 200°C, held for 5 min, then raised at 10°C min^{-1} to 240°C and held for 5 min. H_2 injector and flame-ionization detector temperatures were 230 and 250°C, respectively. FAME were identified by comparison of their retention times with those of authentic standards (Sigma), and quantified using methylnonadecanoate as an internal standard. Peaks were integrated with an HP model 3392A.

TABLE 1
Mycelial Dry Weight of Four-Day-Old Liquid Cultures of *Cladosporium cucumerinum, Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL), and *Sporothrix flocculosa* Grown in Absence (control) or Presence (treated) of Antifungal Fatty Acids Produced by *S. Flocculosa*

Fungus	Control[a] (mg)	Treated (mg)	% Inhibition
C. cucumerinum	913	446	51
FORL	1065	712	33
S. flocculosa	836	796	5

[a]Values are means of three different cultures.

The degree of unsaturation was determined as previously described (20) where:

$$\Delta/\text{mole} = [\%18{:}1 + 2\,(\%18{:}2) + 3\,(\%18{:}3)]/100 \quad [1]$$

Sterols were estimated by the method of Zlatkis *et al.* (21), using ergosterol (Sigma) as standard.

Preparation of liposomes. Liposomes were prepared according to the method of Alving *et al.* (22). Briefly, total lipids extracted from *S. flocculosa* and *C. cucumerinum* were dissolved separately in chloroform/methanol (2:1; vol/vol) and transferred to round-bottom flasks. Under a gentle flow of nitrogen, the solvent was evaporated to dryness to form a thin lipid film on the walls of the flasks. Liposomes were prepared by dispersing the lipids in 10 mM Tris-HCl buffer, pH 7.4, containing 145 mM NaCl, followed by vortexing for 10 min in the presence of glass beads. To the final liposome preparation, 1,6-diphenyl-1,3,5-hexatriene (DPH) was added to give 1 mM.

Fluorescence anisotropy. Fluorescence measurements were taken with a SLM spectrofluorimeter (Aminco; SLM instruments, Urbana, IL) equipped with Glan-Thompson polarization optics with excitation and emission wavelengths of 360 and 430 nm, respectively. Anisotropy values (r) were determined as described earlier (24) where $r = I_H - I_V/I_H + 2\,I_V$; I_H and I_V being the intensity of horizontal (parallel) and vertical (perpendicular) polarized light, respectively. The standard deviation of polarization values was 0.02.

RESULTS

General effects on growth. As previously reported (8), all three fatty acids exhibited antifungal activity on the TLC-bioassay when an equivalent of roughly 7.5 μg of each molecule was spotted on the plates. By contrast, only a faint zone of activity was detected with oleic acid when 1000 μg were spotted.

The three fungi used in this study varied considerably in their sensitivity to the fatty acids. For instance, at the concentration used in this study (0.4 mg/mL), the antibiotics inhibited the growth of *C. cucumerinum* and *F. oxysporum* by 51 and 33%, respectively, whereas growth of *S. flocculosa* was only slightly inhibited (Table 1).

Total lipid composition. Separation of total lipids by TLC revealed no qualitative differences in lipid classes among the fungi tested (Table 2). However, quantitative comparison of total lipids indicated that *C. cucumerinum* had a lower relative cellular lipid content than *S. flocculosa* or FORL. After treatment with the antifungal fatty acids, the total lipids in *C. cucumerinum* and FORL were decreased by 18 and 11%, respectively, although they remained unchanged in *S. flocculosa* (Table 2).

Effects on sterols and individual phospholipids. The total sterol content in *C. cucumerinum* was much lower than in *S. flocculosa* or FORL controls (Table 2). Although there was no major effect of antibiotics on the total sterol content in the three fungi, cellular phospholipids decreased by 30 and 19% in *C. cucumerinum* and FORL, respectively (Table 2). In *S. flocculosa*, no apparent effect on total phospholipids was detected. For the sterol/phospholipid ratio (S/P), it increased to a higher extent in both *C. cucumerinum* and FORL than in *S. flocculosa* (Table 2).

The proportion of individual phospholipids varied among the three fungi (Table 3). In *S. flocculosa* and *C. cucumerinum* phosphatidylcholine (PC) and phosphatidylethanolamine (PE) together represented more than 70% of total phospholipids. Nevertheless, the PC/PE ratio in *C. cucumerinum* was much higher (3.1) than in *S. flocculosa* (1.7). In FORL the proportion of phosphatidylinositol and phosphatidylserine was higher compared to the other two fungi (Table 3). Finally, the cardiolipin content was considerably higher in *C. cucumerinum* (Table 3).

After treatment with the antifungal fatty acids, the most important quantitative differences were a 4- and 2-fold increase in phosphatidic acid (PA) in *C. cucumerinum* and in FORL, respectively (Table 3). Comparatively, the differences between levels of each phospholipid were negligible between control and treated *S. flocculosa*.

Effect on fatty acid composition. As shown in Table 2, free fatty acid contents were five and three times higher in *C. cucumerinum* and FORL, respectively, following treatment with antifungal fatty acids. In *S. flocculosa*, the amount of cellular free fatty acids was slightly increased.

Among phospholipid fatty acids, palmitic and oleic acids were the most prevalent saturated and unsaturated fatty acids,

TABLE 2
Lipid Composition in *Sporothrix flocculosa*, FORL[a], and *Cladosporium cucumerinum* Before (control) and After (treated) Exposure to a Subinhibitory Dose of Antifungal Fatty Acids Produced by *S. flocculosa*

	S. flocculosa		FORL		*C. cucumerinum*	
	Control[b,c]	Treated	Control	Treated	Control	Treated
Total lipids	43.1 ± 1.5	44.6 ± 1.2	39.5 ± 1.5	35.7 ± 1.4	20.6 ± 0.9	16.9 ± 1.1
Sterols	7.9 ± 0.6 (19)	8.9 ± 0.6 (19)	8.9 ± 0.1 (23)	8.7 ± 0.1 (24)	3.1 ± 0.1 (15)	3.0 ± 0.1 (18)
Free fatty acids	2.7 ± 0.1 (6)	3.2 ± 0.0 (7)	2.8 ± 0.0 (7)	7.2 ± 0.1 (20)	0.9 ± 0.0 (4)	3.7 ± 0.1 (22)
Phospholipids	14.6 ± 1.1 (34)	14.3 ± 1.1 (31)	17.8 ± 1.3 (45)	12.8 ± 1.4 (36)	10.8 ± 1.2 (53)	6.3 ± 0.1 (37)
S/P ratio[d]	0.5	0.6	0.5	0.6	0.3	0.5

[a]See Table 1 for abbreviation.
[b]Values are means ± SD of three different batches.
[c]Values are expressed as percentage of the total dry weight. Values in parentheses are expressed as percentage of total lipids.
[d]Sterol/phospholipid ratio.

TABLE 3
Glycerophospholipid Composition in *Sporothrix flocculosa*, FORL[a], and *Cladosporium cucumerinum* Before (control) and After (treated) Exposure to a Subinhibitory Dose of Antifungal Fatty Acids Produced by *S. flocculosa*

	% of Total phospholipids					
	S. flocculosa		FORL		*C. cucumerinum*	
	Control[b,c]	Treated	Control	Treated	Control	Treated
PA	1.7 ± 0.0	1.8 ± 0.0	2.0 ± 0.4	4.1 ± 1.1	0.5 ± 0.1	2.2 ± 1.1
PE	32.1 ± 2.1	29.9 ± 3.1	27.6 ± 1.9	23.9 ± 3.0	18.5 ± 2.0	15.2 ± 2.1
PI + PS	10.6 ± 1.1	11.2 ± 1.1	26.7 ± 2.1	29.3 ± 2.3	2.3 ± 0.9	2.2 ± 1.1
PC	53.6 ± 3.2	55.1 ± 2.9	29.2 ± 3.4	26.6 ± 2.7	56.3 ± 3.3	47.7 ± 1.1
CL	1.3 ± 0.3	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	16.2 ± 3.1	15.3 ± 2.7
Unknown at solvent front	2.3 ± 0.1	2.9 ± 0.1	5.5 ± 0.1	4.2 ± 0.1	1.3 ± 0.1	2.3 ± 0.1

[a]See Table 1 for abbreviation; PA, phosphatidic acid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; CL, cardiolipin..
[b]Values are means ± SD of three different batches.
[c]Values are expressed as percentage of the total phospholipids.

TABLE 4
Effects of a Subinhibitory Dose of Antifungal Fatty Acids Produced by *Sporothrix flocculosa* on Phospholipid Fatty Acid Content (as relative percentage) of *S. flocculoisa*, FORL[a], and *Cladosporium cucumerinum*

	S. flocculosa		FORL		*C. cucumerinum*	
Fatty acid	Control	Treated	Control	Treated	Control	Treated
16:0	14.8	11.6	13.1	10.6	11.6	10.1
18:0	6.3	10.4	9.2	6.7	3.1	9.7
18:1	42.4	43.7	42.5	36.6	49.9	38.6
18:2	26.4	27.4	28.5	36.9	37.1	49.8
18:3	3.5	3.5	4.0	8.0	4.6	10.9
18:1 (18:2 + 18:3)	1.4	1.4	1.4	0.8	1.6	0.6
Δ/mol	1.1	1.1	1.2	1.4	1.4	1.7

[a]See Table 1 for abbreviations.

respectively, in the three fungi used in this study (Table 4). The degree of unsaturation (Δ/mole) was higher in *C. cucumerinum* than in FORL and *S. flocculosa* controls (Table 4). After treatment with the antibiotics the degree of unsaturation remained stable in *S. flocculosa*, and it increased in *C. cucumerinum* and FORL by 22 and 13%, respectively (Table 4). Accordingly, the ratio was considerably reduced in *C. cucumerinum* and FORL following treatment, due to the decreased proportion of 18:1 and increased 18:2 and 18:3, respectively. In the case of phospholipids from *S. flocculosa*, the ratio was unchanged in response to the treatment (Table 4).

Fluorescence polarization. The effect of fungitoxic fatty acids on lipid fluidity was investigated using DPH, an intrinsic fluorescent probe located deep within the hydrophobic core of the membrane bilayer of liposomes. The fluorescence anisotropy or *r* value is taken as an index of membrane fluidity, with decreasing *r* values indicating increases in lipid fluidity. Anisotropy values from lipids built with lipids of *C. cucumerinum* were lower than the ones recorded with liposomes from *S. flocculosa* lipids (Table 5). Additionally, the fungitoxic fatty acids caused dose-dependent decreases in anisotropy (*r*) values in *C. cucumerinum*, thus increasing lipid fluidity by more than 100% at the highest concentration tested (0.5 mg/mL). By contrast, *r* values in liposomes from lipids of *S. flocculosa* only indicated a 17% increase in lipid fluidity at the same concentration (Table 5).

TABLE 5
Fluidity (*r*) of Liposomes Prepared with Lipids Extracted from Liquid Cultures of *Cladosporium cucumerinum* and *Sporothrix flocculosa* Grown in the Presence of Increasing Concentrations of Antifungal Fatty Acids Produced by *S. flocculosa*

	$r^{a,b}$	*r*
Concentrations (mg/mL)	*C. cucumerinum*	*S. flocculosa*
0	0.19 ± 0.03	0.28 ± 0.03
0.2	0.15 ± 0.01	0.29 ± 0.07
0.4	0.11 ± 0.02	0.27 ± 0.03
0.5	0.09 ± 0.01	0.24 ± 0.04

[a]Values are means ± SD of three different preparations of liposomes.
[b]*r* represents anisotropy values where $r = I_H - I_V / I_H + 2\ I_V$; I_H and I_V being the intensity of horizontal and vertical polarized light, respectively.

DISCUSSION

Lipids are major components of the cell membrane and many metabolic reactions occur in association with these membranes. The physical properties of lipids and the dynamic nature of membrane have important effects on the function of

many membrane-associated proteins. The modification of the lipidic environment in membranes may therefore be harmful to cell viability (24,25). Several antimicrobial molecules have been found to interfere with lipid metabolism or directly with specific lipid molecules (26,27). Recently, new fatty acids with antifungal activity were isolated and characterized from liquid cultures of the biocontrol agent *S. flocculosa* (6,7). Cytochemical and physiological studies revealed that these antibiotics induced a dysfunction of the cytoplasmic membrane. Because these antifungal molecules seem to have a differential effect on fungi, three fungi, with different sensitivity to antibiotics, were selected for the purpose of this study: *C. cucumerinum*, sensitive, FORL, moderately sensitive, and *S. flocculosa*, resistant (5,6). The analysis of lipid composition among the three fungi, both before and after treatment with antibiotics, revealed some important differences which appear to be related to their level of sensitivity.

Firstly, *C. cucumerinum* had a much lower lipid content than the two other fungi. This low concentration of lipids had been previously reported with another species of *Cladosporium* (25), and may be related to the sensitivity of this fungus to the antifungal fatty acids. Additionally, *C. cucumerinum* contains less sterols than FORL or *S. flocculosa*. This factor could also play a role in its greater sensibility to the antibiotics, because it is well established that the presence in a lipid bilayer of sterols tends to buffer the bilayer against stress-induced fluctuation in fluidity (28). Another interesting observation was the degradation of phospholipids in both sensitive fungi (*C. cucumerinum* and FORL) following treatment. This breakdown of phospholipids was correlated with a drastic increase in free fatty acid contents. Free fatty acids are known to be toxic in high concentrations (26), and the increase observed as a result of exposure to the antibiotics may explain their higher sensitivity. The accumulation of these fatty acids could not only be attributed to an increase in breakdown of acyl lipid, but also to a reduction in fatty acid incorporation into acyl lipids and/or a stimulation of *de novo* synthesis, as a consequence of the disparity between fatty acid synthesis and utilization.

For the individual phospholipids, further differences were noted between the three fungi before and after treatment, and sometimes with a correlation to sensitivity to antifungal fatty acids. It is well known that head groups, charge and structure of phospholipids, and the fluctuation in their levels may alter membrane permeability and membrane-associated functions (29,30). Indeed, previous studies have shown that PE molecules are more closely packed than PC molecules (31). In the sensitive fungus (*C. cucumerinum*), the PC/PE ratio was much higher than in *S. flocculosa*. In addition, *C. cucumerinum* contains a high quantity of cardiolipin in its lipids. This phospholipid has the highest transition temperature of all lipids at neutral pH (32). However, it cannot be directly involved in the alteration of plasma membrane function as this lipid is characteristically located in the inner membrane of mitochondria. The increases of PA molecules in sensitive fungi, following treatment, is therefore probably correlated with a degradation of phospholipids.

Another feature shared by *C. cucumerinum* and FORL was their higher level of phospholipid unsaturated fatty acids compared to *S. flocculosa*. This higher level, combined with an increase in unsaturation following treatment with antibiotics, could be involved in the alteration of plasma membrane function in sensitive fungi, as previously reported (6). Indeed, changes in the degree of unsaturation of phospholipids will alter the fluidity of the membrane, i.e., the degree of mobility of the phospholipid fatty acyl chains in the bilayer. Changes in unsaturation have also been reported to affect the activity of membrane-bound enzymes and proteins (33). The involvement of the desaturase activities in the increase of unsaturation of fatty acids has been previously demonstrated (34). These desaturases are lipophilic microsomal enzymes; their activities are modulated by the fluidity of the membrane in which they are embedded (35).

The results concerning the degradation of phospholipids, the differences in head groups of phospholipids, and the increases in unsaturation of phospholipid fatty acids suggest an alteration in membrane environment. To address this question, the effects of antifungal fatty acids on lipid fluidity were determined by fluorescence anisotropy measurement using liposomes and the probe DPH. The low value of anisotropy of liposomes built with lipids from untreated *C. cucumerinum* could be explained by a constitutive high degree of unsaturation of phospholipid fatty acids, a low quantity of sterols, and the presence of a high proportion of cardiolipin, as described previously. The drastic decrease in anisotropy values in liposomes prepared with lipids from treated *C. cucumerinum* was consistent with modifications observed such as the increase in phospholipid fatty acids unsaturation and the degradation of phospholipids. In addition, the increase in lipid fluidity reflects an increase in membrane lipid disorder, which is coherent with the reported deterioration of cell membranes and enhanced permeability induced by metabolites from *S. flocculosa* (6).

In conclusion, fungal sensitivity to fatty acid antibiotics from *S. flocculosa* appears to be related to a high degree of unsaturation of phospholipid fatty acids and a low proportion of sterols. The major responses of sensitive fungi to sublethal doses of antifungal fatty acids from liquid culture of *S. flocculosa* were: (i) a decrease in total lipid; (ii) an increase in the degree of fatty acid unsaturation (18:1 > 18:2 > 18:3); (iii) an increase in free fatty acids and PA, and a decrease in total phospholipids; and (iv) an increase in S/P ratio. These modifications in lipid composition led to an increase in membrane fluidity, which is responsible for the previously demonstrated alteration of membrane structure and function in fungi confronted to *S. flocculosa* (5) and its toxic metabolites (6).

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Ultrasound-Assisted Synthesis of Santalbic Acid and a Study of Triacylglycerol Species in *Santalum album* (Linn.) Seed Oil

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ABSTRACT: Methyl ricinoleate (**1**) was treated with bromine and the dibromo derivative (**2**) was reacted with ethanolic KOH under ultrasonic irradiation to give 12-hydroxy-octadec-9-ynoic acid upon acidification with dil. HCl. The latter compound was methylated with BF_3/methanol to give methyl 12-hydroxy-octadec-9-ynoate (**3**). Compound **3** was treated with methanesulfonyl chloride in the presence of triethylamine in CH_2Cl_2 to give methyl 12-mesyloxy-octadec-9-ynoate (**4**). Reaction of methyl 12-mesyloxy-octadec-9-ynoate with aqueous KOH under ultrasonic irradiation (20 kHz) gave (11*E*)-octadecen-9-ynoic acid (**5**, santalbic acid, 40%) and (11*Z*)-octadecen-9-ynoic acid (**6**, 60%) on acidification with dil. HCl. These isomers were separated by urea fractionation. The ^{13}C nuclear magnetic resonance (NMR) spectroscopic properties of the methyl ester and the triacylglycerol (TAG) esters of these enynoic fatty acid isomers were studied. The carbon shifts of the unsaturated carbon nuclei of the methyl ester of the *E*-isomer were unambiguously assigned as 88.547 (C-9), 79.287 (C-10), 109.760 (C-11), and 143.450 (C-12) ppm, while the unsaturated carbon shifts of the (*Z*)-enynoate isomer appeared at 94.277 (C-9), 77.561 (C-10), 109.297 (C-11), and 142.668 (C-12) ppm. In the ^{13}C NMR spectral analysis of the TAG molecules of type AAA containing either the (*Z*)- or (*E*)-enyne fatty acid, the C-1 to C-6 carbon atoms on the α- and β-acyl positions were differentiated. The unsaturated carbon atoms in the α- and β-acyl chains were also resolved into two signals except that of the C-11 olefinic carbon. Sandal *(Santalum album)* wood seed oil (a source of santalbic acid) was separated by silica chromatography into three fractions. The least polar fraction (7.2 wt%) contained TAG which had a random distribution of saturated and unsaturated fatty acids, of which oleic acid (69%) was the predominant component. The second fraction (3.8 wt%) contained santalbic acid (58%) and oleic acid (28%) together with some other normal fatty acids. Santalbic acid in this fraction was found in both the α- and β-acyl positions of the glycerol "backbone." The most polar fraction (89 wt%) consisted of TAG containing santalbic acid only. The distribution of the various fatty acids on the glycerol "backbone" was supported by the results from the ^{13}C NMR spectroscopic analysis.

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The chemistry of conjugated long-chain fatty alcohols and esters containing an enyne system is of current interest for the synthesis of biologically active compounds such as pheromones, leukotrienes, and prostaglandins (1,2). The seed oil of *Santalum album* (Sandalwood) contains a significant level of (11*E*)-octadecen-9-ynoic acid (commonly known as santalbic or ximenynic acid) (79 wt%) (3). Sandal seed oil is used in the treatment of skin diseases and shows antiviral and diuretic activities (4). The plant *Ixiolaena brevicompta*, which contains acetylenic fatty acids, is reported to be responsible for causing acute muscular degeneration and mortalities in sheep. These mortalities have been linked to the inhibitory effects of acetylenic acids on cyclooxygenase, lipoxygenase, and phospholipase A_2 activities (5–7). Spitzer *et al.* (8) have also reported the occurrence of santalbic (ximenynic) acid in the seed oil of *Curupira tefeensis* (Fam. Olacaceae). Pasha and Ahmad (3) have recently described the preparation of various oxygenated fatty acid esters from the methyl ester of natural santalbic acid.

The total synthesis of santalbic acid has been described by Crombie and Jacklin (9) from acetylenic intermediates, which required seven steps. Gunstone and Said (10) have reported a mixture of methyl santalbate (38%) and a methylene-interrupted enyne derivative [viz. methyl (12*E*)-octadecen-9-ynoate] (57%) as the main products when methyl 12-mesyloxy-octadec-9-ynoate is refluxed with NaOMe in methanol. Despite the abundant occurrence of triacylglycerols (TAG) containing acetylenic acids in many seed lipids, little work has been carried out to analyze their molecular species. The only information on the distribution of a methylene-interrupted enyne fatty acid [viz. crepenynic acid, (9*Z*)-octadecen-12-ynoic acid] has been reported for the seed oil of *Crepis rubra* (11). In this seed oil, crepenynic acid is found to be mainly esterified to the glycerol at the *sn*-2 and *sn*-3 positions with saturated acids occupying mainly the *sn*-1 position.

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Abbreviations: GLC, gas–liquid chromatography, IR, infrared spectroscopy, NMR, nuclear magnetic resonance spectroscopy, R_f, retardation factor, TAG, triacylglycerol, TLC, thin-layer chromatography.

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In this paper we describe (i) an efficient ultrasound-assisted synthesis of santalbic acid and its (*Z*)-isomer starting from ricinoleic acid [12-hydroxy-(9*Z*)-octadecenoic acid] *via* the mesyloxy derivative of an acetylenic intermediate (methyl 12-hydroxy-octadec-9-ynoate) and (ii) the analysis of TAG molecular species in Sandalwood (*Santalum album*) seed oil by ^{13}C nuclear magnetic resonance (NMR) spectroscopy.

MATERIALS AND METHODS

The matured seeds of *S. album* (Sandalwood) were supplied by Sandal Research Centre (Bangalore, India). Analytical grade reagents and adsorbents (silica gel GF_{254}) for thin-layer chromatography (TLC) and column chromatography were obtained from E. Merck (Darmstadt, Germany). Porcine pancreatic lipase was purchased from Sigma Chemical Co. (St. Louis, MO). Gas–liquid chromatographic (GLC) and ^1H and ^{13}C NMR spectroscopic analyses were conducted by methods described elsewhere (12). Extraction of the seed oil, transmethylation, and pancreatic lipase hydrolysis followed by methylation for the determination of the fatty acid composition were performed according to the methods described by Pasha and Ahmad (3,13). Ultrasonication was carried out using a 20 kHz (53 W/cm^2) ultrasound horn (Undatim Ultrasonic S.A., Louvain-la-neuve, Belgium) with the reaction mixtures contained in a water-cooled cell at 20°C. The preparations of TAG of type AAA using santalbic acid or its (*Z*)-isomer were conducted as described by Ziegler and Berger (14).

EXPERIMENTAL PROCEDURES

Preparative TLC fractionation of Sandalwood seed oil. The seed oil (200 mg) was dissolved in *n*-hexane (20 mL), and the solution was applied on 10 silica gel coated TLC plates (0.3 mm thick). The plates were developed four times each (multiple developments) in a mixture of *n*-hexane/diethyl ether/acetic acid (85:15:5, vol/vol) at 5°C. The plates were viewed under ultraviolet light and bands corresponding to retardation factor (R_f) = 0.6 (fraction A), 0.5 (fraction B), and 0.3–0.4 (fraction C) were scraped off. The combined bands of each fraction of silica were extracted with diethyl ether (4 × 20 mL) to give fractions A (14.4 mg), B (7.6 mg), and C (176 mg). The isolated samples of TAG fractions were stored under nitrogen in *n*-hexane at 0–5°C.

*Methyl 9,10-dibromo-12-hydroxyoctadecanoate (**2**).* Bromine (5.6 g, 3.5 mmol) was added to a solution of methyl ricinoleate (**1**, 2.0 g, 6.4 mmol) in diethyl ether (30 mL) and stirred for 15 min. The reaction mixture was successively washed with aqueous sodium thiosulfate solution (5%, 10 mL), water (20 mL), and dried over anhydrous sodium sulfate. The ethereal mixture was filtered and the filtrate evaporated to give crude methyl 9,10-dibromo-12-hydroxyoctadecanoate (**2**, 3.0 g, 100%) as a viscous liquid. TLC, R_f 0.6 (petroleum ether/diethyl ether, 7:3, vol/vol); infrared (IR) (cm^{-1}): 3450 (*br*), 1740(*s*), 840(*m*), 760 (*m*), and 720(*s*); ^1H NMR ($CDCl_3$, δ) 0.88(*t*, 3H, CH_3), 1.2–1.8 (*m*, 20H, CH_2), 1.9-2.1(*m*, 4H, CH_2), 2.2(*s*, 1H, CH-O*H*), 2.3(*t*, 2H, 2-*H*), 3.6(*s*, 3H, $COOCH_3$), 3.8(*m*, 1H, C*H*-OH), and 4.6(*m*, 2H, C*H*-Br).

*Methyl 12-hydroxy-octadec-9-ynoate (**3**).* A mixture of compound **2** (3.0 g, 6.3 mmol), ethanol (95%, 30 mL), and KOH (5.3 g, 9.5 mmol) was sonicated for 30 min using a 20 kHz horn at 20°C. The reaction mixture was acidified with HCl (6M, 20 mL) and extracted with diethyl ether (3 × 30 mL). The ethereal extract was washed with water (30 mL) and dried over anhydrous sodium sulfate. The filtrate was evaporated, and the residue was refluxed with borontrifluoride/methanol (14%, w/w, 5 mL) in absolute methanol (30 mL) for 10 min. Water (50 mL) was added and the reaction mixture was extracted with diethyl ether (2 × 50 mL). The solvent was evaported, and silica column chromatographic purification of the residue using a mixture of petroleum ether and diethyl ether (85:15, vol/vol) as eluent gave pure methyl 12-hydroxy-octadec-9-ynoate (**3**, 1.3 g, 66%). TLC, R_f 0.5 (petroleum ether/diethyl ether, 7:3, vol/vol); IR (cm^{-1}): 3450 (*br*), 1740(*s*), 1460(*m*), 1435(*m*), 1360(*m*), and 1170(*m*); ^1H NMR ($CDCl_3$, δ): 0.88 (*t*, 3H, CH_3), 1.2–1.6 (*m*, 20H, CH_2), 2.04 (*s*, 1H, CH-O*H*), 2.2 (*m*, 4H, 8-*H* and 11-*H*), 2.3 (*t*, 2H, 2-*H*), 3.66 (*s*, 3H, $COOCH_3$), and 3.96 (*m*, 1H, C*H*-OH); ^{13}C NMR ($CDCl_3$, ppm): 14.1 (C-18), 18.7 (C-8), 22.6 (C-17), 24.9 (C-3), 25.2 (C-14), 27.7 (C-11), 28.6 (C-7), 28.9, 29.3, 31.8 (C-16), 33.9 (C-2), 36.2 (C-13), 51.4 ($COOCH_3$), 70.3 (C-12), 76.2 (C-10), 83.1 (C-9), and 174.3 (C-1)

*Methyl 12-mesyloxy-octadec-9-ynoate (**4**).* A mixture of compound **3** (1.0 g, 3.2 mmol), methanesulfonyl chloride (0.8 g, 7.0 mmol), triethylamine (1.0 mL) and dichloromethane (10 mL) was stirred at 0–5°C for 30 min. The reaction mixture was diluted with dichloromethane (50 mL) and successively washed with dilute HCl (2M, 5 mL), water (20 mL), and dried over anhydrous sodium sulfate. The mixture was filtered and the filtrate evaporated to give crude methyl 12-mesyloxy-octadec-9-ynoate (**4**, 1.2 g, 95%) as a viscous oil. TLC, R_f 0.4 (petroleum ether/diethyl ether, 1:1, vol/vol), IR (cm^{-1}): 1735 (*s*), 1440 (*m*), 1460 (*m*), 1360 (*s*), 1170 (*s*), and 910 (*s*); ^1H NMR ($CDCl_3$, δ) 0.88 (*t*, 3H, CH_3), 1.2–1.6 (*m*, 18H, CH_2), 2.1 (*t*, 3H, 8-*H*), 2.3 (*t*, 2H, 2-*H*), 2.6 (*d*, 2H, 11-*H*), 3.05 (*s*, 3H, CH_3 of OMs); 3.7 (*s*, 3H, $COOCH_3$), and 4.7 (*quintet*, 1H, C*H*-OMs); ^{13}C NMR ($CDCl_3$, ppm) 14.1 (C-18), 18.6 (C-8), 22.5 (C-17), 24.8 (C-3), 24.9, 25.2 (C-14), 28.6 (C-7), 28.7, 28.8, 28.9, 29.0, 31.6 (C-16), 33.9, 34.0 (C-2), 38.7 (CH_3 of OMs), 51.4 ($COOCH_3$), 74.6 (C-10), 81.5 (C-12), 83.3 (C-9), and 174.2 (C-1).

*(11E)-Octadecen-9-ynoic acid (**5**) and (11Z)-octadecen-9-ynoic acid (**6**).* A mixture of methyl 12-mesyloxy-octadec-9-ynoate (1.0 g, 2.6 mmol), KOH (1.2 g), and water (20 mL) was placed in a water-cooled cell (20°C) and sonicated (20 kHz) for 30 min. The reaction mixture was acidified with dilute HCl (6M, 20 mL) and extracted with diethyl ether (3 × 50 mL). The ethereal extract was washed with brine (30 mL) and dried over anhydrous sodium sulfate. The filtrate was

evaporated to give crude enynoic acids (0.70 g, 97%). Gas chromatography (GC) analysis of the methyl esters of this mixture showed (11*E*)-octadecen-9-ynoic acid (**5**, santalbic acid) and its (*Z*)-isomer (**6**) in the ratio of 2:3.

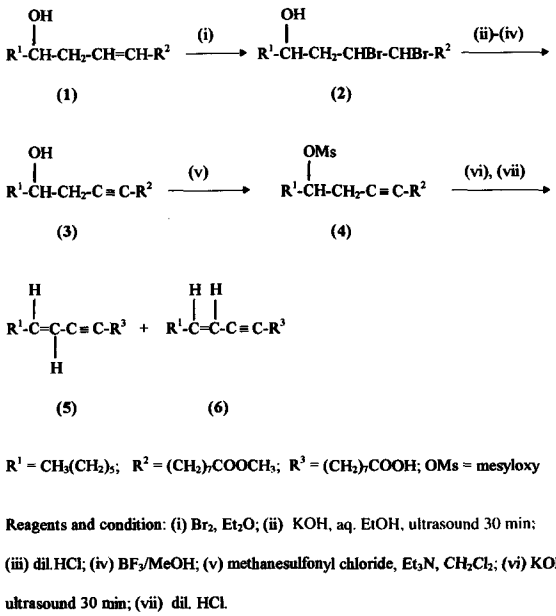
The mixture of the crude acids (0.7 g), urea (3.0 g), and methanol (9 mL) was warmed to dissolve the urea crystals. The solution was allowed to cool to room temperature and was kept at 5°C for 4 h. The solution was filtered to recover the solid urea adduct. The collected urea adduct was dissolved in dilute HCl (2M, 25 mL). The acidic solution was extracted with diethyl ether (3 × 30 mL). The organic extract was washed with water (30 mL) and dried over Na_2SO_4. The filtrate was evaporated and the residue was twice refractionated (collecting the urea adduct each time) by using urea (1 g) and methanol (3 mL), which yielded in pure santalbic acid (**5**, 0.24 g, m.p. 39°C., lit. (15) 39–40°C).

The mother liquor from the first fractionation process was acidified with dilute HCl (2M, 10 mL). The solution was extracted with diethyl ether (3 × 30 mL). The ethereal extract was washed with water (30 mL) and dried (Na_2SO_4). The filtrate was evaporated and repeated (twice) urea fractionation of the mother liquors with urea (1 g) and methanol (3 mL) gave pure (11*Z*)-octadecen-9-ynoic acid (**6**, 0.36 g) as a viscous oil.

*Cleavage oxidation of (11*E*)-octadecen-9-ynoic acid.* A mixture of (11*E*)-octadecen-9-ynoic acid (25 mg, 0.09 mmol), glacial acetic acid (18 mL), water (2 mL), and KMnO4 (0.2 g, 1.3 mmol) was sonicated for 15 min at 20°C. The reaction mixture was diluted with water (20 mL) and extracted with diethyl ether (3 × 10 mL). The ethereal extract was washed with water (20 mL) and dried over anhydrous sodium sulfate. The filtrate was evaporated to give a residue (20 mg). The residue was refluxed with borontrifluoride/methanol (14%, w/w, 2 mL) in absolute methanol (10 mL) for 5 min. Water (20 mL) was added and the reaction mixture was extracted with *n*-hexane (2 × 10 mL). The organic extract was washed with water (5 mL) and dried over anhydrous sodium sulfate. The solvent was evaporated and the resulting methyl esters were analyzed by GLC. GLC analysis showed the esterified products as methyl heptanoate and dimethyl nonanedioate by reference to authentic samples.

RESULTS AND DISCUSSION

*Synthesis and properties of santalbic acid (**5**) and its (Z)-isomer (**6**).* We have made successful use of ultrasound as an energy source in chemical transformations involving various fatty acid molecules (12,16–18 and Lie Ken Jie, M.S.F., and P. Kalluri, submitted for publication). The synthesis route for the preparation of santalbic acid and its (*Z*)-isomer is presented in Scheme 1. The ultrasound-assisted dehydrobromination reaction of methyl 9,10-dibromo-12-hydroxy-octadecanoate (obtained by bromination of methyl ricinoleate) using ethanolic KOH gave 12-hydroxy-octadec-9-ynoic acid (**3**) in high yield (12). The latter compound was esterified and mesylated. Reaction of methyl 12-mesyloxy-octadec-9-ynoate



$R^1 = CH_3(CH_2)_5$; $R^2 = (CH_2)_7COOCH_3$; $R^3 = (CH_2)_7COOH$; OMs = mesyloxy

Reagents and condition: (i) Br_2, Et_2O; (ii) KOH, aq. EtOH, ultrasound 30 min; (iii) dil.HCl; (iv) BF_3/MeOH; (v) methanesulfonyl chloride, Et_3N, CH_2Cl_2; (vi) KOH, H_2O, ultrasound 30 min; (vii) dil. HCl.

SCHEME 1

(**4**) with aqueous KOH under ultrasound (30 min) furnished in almost quantitative yield a mixture of santalbic acid (**5**, 40%) and its (*Z*)-isomer [**6**, (11*Z*)-octadecen-9-ynoic acid, 60%] as determined by GLC. Gunstone and Said (10), however, reported in a similar reaction the isolation of methyl santalbate (38%) and a mixture of the methylene-interrupted methyl (12*Z/E*)-octadecen-9-ynoate isomers, when the same mesyloxy intermediate was refluxed with NaOMe in MeOH. To verify Gunstone's results, the reaction was repeated by refluxing methyl 12-mesyloxy-octadec-9-ynoate(**4**) with NaOMe in MeOH for 4 h. Analysis of the isolated products showed the presence of a mixture of methyl (*E/Z*)-octadecen-9-ynoate (20%), methyl 12-methoxy-9-octadecynoate (5%), and unreacted methyl 12-mesyloxy-9-octadecynoate. No methylene-interrrupted enyne fatty esters [i.e., methyl (12*E/Z*)-octadecen-9-ynoate] were detected in the isolated product mixture. The same reaction, when conducted under ultrasound (15 min), gave a mixture of methyl (*E/Z*)-octadecen-9-ynoate in low yield (20%). We were therefore unable to reproduce the reaction as reported by Gunstone and Said (10). In considering the stability of the two possible enyne systems obtainable from this elimination reaction involving compound **4**, we would expect the conjugated enyne isomer to be preferentially formed over the methylene-interrupted enyne isomers.

The separation of the obtained mixture of enynoic acids (**5,6**) into pure components was achieved by repeated urea fractionation. Pure santalbic acid (**5**, 34 wt%) was isolated after three urea fractionations. The mother liquor yielded pure (11*Z*)-octadecen-9-ynoic acid (**6**, 51 wt%) after three urea fractionations. The position of the enyne system in santalbic acid and its (*Z*)-isomer was determined by a newly developed ultrasound-assisted oxidative cleavage procedure as described elsewhere (Lie Ken Jie, M.S.F., and P. Kalluri, sub-

mitted for publication). This procedure involved the reaction of santalbic acid or its (*Z*)-isomer with $KMnO_4$ in acetic acid/water (9:1, vol/vol) under ultrasonic irradiation (20 kHz) for 15 min. The isolated oxidation products were methylated, and the methyl ester derivatives were identified by GLC analysis as methyl heptanoate and dimethyl nonanedioate. This result confirms the position of the enyne system in compounds **5** and **6**.

The results of the ^1H and ^{13}C NMR analysis of methyl santalbate (**5**) and its (*Z*)-isomer (**6**) are presented in Table 1. The ^1H NMR spectrum of the synthetic methyl santalbate showed the characteristic doublet at δ 5.47 and a multiplet at δ 6.04 for the shifts of the 11-*H* and 12-*H* ethylenic protons, respectively. The multiplet at δ 2.05 was assigned to the shift of the 13-*H* protons, as confirmed by double irradiation technique. The shift of the methylene protons (8-*H*) adjacent to the acetylenic bond appeared at δ 2.26. This signal merged with the triplet of the 2-*H* protons (δ 2.31). In the ^{13}C NMR spectrum of methyl santalbate, the shifts of the C-11 and C-12 ethylenic carbon atoms appeared at δ 109.76 and 143.45, respectively. These chemical shifts were confirmed from the results of the ^1H-^{13}C COSY spectrum. In order to assign the shifts of the acetylenic carbon atoms unequivocally, it was necessary to run a long-range ^1H-^{13}C heteronuclear correlation spectrum optimized at $J_{C\text{-}H}$ 10 Hz. This technique allowed interconnections up to three nuclei to be established. From these results, the shifts of the C-9 and C-10 carbon atoms were confirmed as δ 88.55 and 79.29, respectively. The presence of the (*E*)-enyne system was confirmed by the signal at δ 19.33 for the shift of the C-8 methylene carbon atom and by the signal at δ 32.98 for the shift of C-13.

The ^1H NMR spectrum of methyl (11*Z*)-octadecen-9-ynoate differed significantly from that of the (*E*)-isomer (methyl santalbate) in that the 12-*H* olefinic proton appeared as a distinct double triplet at δ 5.83, while the signal for the 11-*H* protons remained as a doublet at δ 5.44(d). The ^{13}C NMR spectrum of the (*Z*)-isomer showed a significant shift difference for one of the acetylenic carbon atoms. The shift of the C-9 acetylenic carbon (δ 94.28) was comparatively more deshielded than the similarly positioned acetylenic carbon atom of methyl santalbate. The effect of the (*Z*)-enyne system caused the shift of the methylene carbon atom (C-13) adjacent to the (*Z*)-double bond to appear at δ 30.04. This shift value was much higher than that expected of the shift of a methylene carbon atom adjacent to an isolated (*Z*)-double bond, which normally appears at *ca.* δ 27.0 from TMS.

From this study of the NMR analysis of methyl santalbate and its (*Z*)-isomer, we were able to determine unambiguously the shifts of the unsaturated carbon atoms of the enyne system in these fatty ester isomers. In view of our successful qualitative and semi-quantitative analysis of saturated and unsaturated TAG (19–22), the ^{13}C NMR spectral properties of the corresponding TAG of type AAA composed of santalbic acid or its (*Z*)-isomer were studied as presented in Table 2. In both TAG containing the (*E*)-enynoic (**7**) or (*Z*)-enynoic (**8**) acids, the shifts of the C-1 to C-6 carbon atoms in the α- and β-position of the glycerol "backbone" were clearly differentiated into individual signals and were readily identified by referring to carbon chemical shifts reported elsewhere (Lie Ken Jie, M.S.F., and P. Kalluri, submitted for publication). The shifts of the acetylenic carbon atoms were also differentiated where the shift value of the C-9 carbon atom in the α-acyl chain appeared higher than that of the C-9 in the β-acyl chain. However, the shift of the acetylenic C-10 carbon shift in the β-acyl chain was higher than that of the α-acyl chain. The olefinic carbon atoms at C-11 in the α- and β-acyl chains were not differentiated, but the C-12 olefinic carbons in the α- and β-acyl chains were separated into two distinct signals. It was therefore of interest to note that in such TAG molecules containing an enyne system, the olefinic carbon atom immediately adjacent to the acetylenic bond remained undifferentiated. This particular observation should be borne in mind when mixtures of TAG containing such conjugated enyne systems were to be analyzed by ^{13}C NMR spectroscopy.

Analysis of the TAG species in sandalwood seed oil. The total fatty acid composition of the TAG of sandalwood seed oil is presented in Table 3, which shows oleic acid (18%) and santalbic acid (79%) as the major fatty acid components by

TABLE 1
^1H and ^{13}C Nuclear Magnetic Resonance Chemical Shift Values of Methyl (11*E*)-octadecen-9-ynoate (5) and Methyl (11*Z*)-octadecen-9-ynoate (6)

Compound		$COOCH_3$	2-*H*	3-*H* - 6-*H*	7-*H*	8-*H*	11-*H*	12-*H*	13-*H*	14-*H*	15-*H* - 17-*H*	18-*H*
5	δ_H	3.67 (*s*)	2.31 (*t*)	[a]	[b]	2.26 (*t*)	5.47 (*d*)	6.04 (*dt*)	2.05 (*q*)	[b]	[a]	0.88 (*t*)
6	δ_H	3.67 (*s*)	2.31 (*t*)	[a]	[c]	231 (*t*)	5.44 (*d*)	5.83 (*dt*)	2.31 (*t*)	[c]	[a]	0.89 (*t*)

Compound		$COOCH_3$	C-1	C-2	C-3	C-4	C-5 C-6 C-7	C-8	C-9	C-10	C-11	C-12	C-13	C-14 C-15	C-16	C-17	C-18
5	δ_C	51.463	174.313	34.074	24.921	29.030	[d]	19.329	88.547	79.287	109.760	143.450	32.980	[d]	31.690	22.614	14.103
6	δ_C	51.447	174.267	34.074	24.937	29.062	[e]	19.512	94.277	77.561	109.297	142.668	30.040	[e]	31.706	22.645	14.119

[a]1.26–1.50 (*m*); [b]1.46–1.58 (*m*); [c]1.53–1.56 (*m*);[d]28.711–28.879; [e]28.696–28.910.

TABLE 2
^{13}C Nuclear Magnetic Resonance Chemical Shifts (ppm) of Triacylglycerols of Type AAA Containing Santalbic Acid (7) and Its Z-isomer (8)

(7)



(8)

$R^1 = CH_3(CH_2)_5CH{=}\underset{E}{CH}C{\equiv}C(CH_2)_7$; $R^2 = CH_3(CH_2)_5CH{=}\underset{Z}{CH}C{\equiv}C(CH_2)_7$

Compound	Glyceryl carbons	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10	C-11	C-12	C-13	C-14	C-15	C-16	C-17	C-18
7	62.091[a]	173.142	33.974	24.806	28.978	28.814	28.783			88.444	79.304		143.296						
								28.786	19.325			109.826		32.988	28.814	28.710	31.697	22.617	14.103
	68.915[b]	172.774	34.138	24.837	28.936	28.842	28.710			88.418	79.319		143.306						
8	62.101	173.179	33.977	24.806	29.016	28.893	28.817			94.224	77.482		142.618						
								28.691	19.500			109.300		30.031	28.817	28.691	31.701	22.636	14.130
	68.901	172.771	34.142	24.840	28.973	28.874	28.805			94.193	77.489		142.602						

[a]Chemical shift values in the α-acyl chains. [b]Chemical shift values in the β-acyl chain.

TABLE 3
Fatty Acid Composition (wt%) of Total TAG and of Fractions (A–C) of *Santalum album* Seed Oil After Transmethylation as Determined by GLC

Fatty acids (wt%)	16:0	16:1	18:0	18:1	18:2	18:3	18:2(9*a*, 11*E*)
Total TAG[a]	0.8	0.6	0.4	18.0	0.7	0.5	79.0
Fraction A	9.2	5.4	1.5	69.6	6.2	8.1	—
α-acyl[b]	10.6	7.3	0.5	65.5	8.3	7.8	—
β-acyl[b]	6.6	2.7	5.4	78.8	2.1	4.4	—
Fraction B	3.6	0.6	3.6	28.2	1.5	3.7	58.8
α-acyl[b]	5.5	0.9	5.5	22.7	1.6	3.6	60.2
β-acyl[b]	—	—	—	39.1	1.3	3.5	56.1
Fraction C	—	—	—	—	—	—	100.0

[a]TAG, triacylglycerols.
[b]Fatty acid composition at β-position was determined by lipolysis with pancreatic lipase; fatty acid composition at α-position was calculated from the total fatty acid composition at α- and β-positions.

GLC analysis of the methyl esters. The oil (a mixture of TAG) was separated by preparative silica TLC into three fractions (A–C). Fraction A (7.2%, w/w) was the least polar fraction, which was followed by fractions B (3.8%, w/w) and C (89%, w/w). The total fatty acid composition and that of each fraction (A–C) are given in Table 3 by GLC analysis of the methyl esters.

Fraction A was composed of saturated fatty acids and 18:1 (69%), 18:2 (6%), and 18:3 (8%). This fraction contained no santalbic acid. The ^{13}C NMR spectrum of fraction A showed a rather random distribution of saturated and unsaturated acyl moieties on the glycerol "backbone" as indicated by the shifts of the C-1 to C-3 and the unsaturated carbon atoms (Table 4). From the intensities of the signals of the unsaturated carbon atoms (C-9 and C-10), the amount (in terms of molar equiva-

TABLE 4
Carbon Chemical Shift Values (ppm) of C-1, C-2, C-3, and the Unsaturated Carbon Atoms of Triacylglycerols Contained in Fraction A

Acyl moiety (acyl position)	Carbon number	Chemical shift	Relative intensity
16:0,18:0 (α)	C-1	173.321	0.97
18:1,18:2,18:3 (α)	C-1	173.287	3.58
18:1,18:2,18:3 (β)	C-1	172.872	1.47
18:1 (α)	C-2	34.205	3.15
18:2,18:3 (β)	C-2	34.164	1.39
16:0,18:0 (α)	C-2	34.063	2.18
18:1 (β)	C-2	34.040	7.68
18:2,18:3 (α)	C-2	34.001	1.35
16:0,18:0 (α)	C-3	24.895	4.03
18:1 (β)	C-3	24.874	2.58
18:2 (α)	C-3	24.853	8.93
18:3 (α)	C-3	24.840	2.52
18:1 (α)	C-3	24.803	1.79
18:1 (β)	C-10	130.039	3.95
18:1 (α)	C-10	130.025	8.54
18:1 (α)	C-9	129.720	7.30
18:1 (β)	C-9	129.693	3.46

TABLE 5
Carbon Chemical Shift Values (ppm) of C-1, C-2, C-3, and the Unsaturated Carbon Atoms of Triacylglycerols Contained in Fraction B

Acyl moiety (acyl position)	Carbon number	Chemical shift	Relative intensity
16:0,18:0,18:1 (α)	C-1	173.264	6.48
18:2(9*a*,11*E*) (α)	C-1	173.226	11.87
16:0,18:0,18:1 (β)	C-1	172.855	2.74
18:2(9*a*,11*E*) (β)	C-1	172.817	7.92
18:1 (β)	C-2	34.207	3.36
18:2(9*a*,11*E*) (β)	C-2	34.168	8.98
18:1 (α)	C-2	34.042	7.57
18:2(9*a*,11*E*) (α)	C-2	34.004	16.07
16:0,18:0 (β)	C-3	24.905	3.72
18:1 (β)	C-3	24.886	4.29
!8:1 (α)	C-3	24.863	9.72
18:2(9*a*,11*E*) (β)	C-3	24.852	12.95
18:2(9*a*,11*E*) (α)	C-3	24.818	17.60
18:1 (α)	C-9	129.722	8.41
18:1 (β)	C-9	129.699	3.92
18:1 (α)	C-10	130.011	10.06
18:1 (β)	C-10	130.035	4.68
18:2(9*a*,11*E*) (α)	C-9	88.490	9.02
18:2(9*a*,11*E*) (β)	C-9	88.461	8.05
18:2(9*a*,11*E*) (α)	C-10	79.299	9.63
18:2(9*a*,11*E*) (β)	C-10	79.311	6.86
18:2(9*a*,11*E*) (α,β)	C-11	109.782	29.98
18:2(9*a*,11*E*) (α)	C-12	143.415	92.92
18:2(9*a*,11*E*) (β)	C-12	143.422	69.48

lent) of the oleic acid in the β-position appeared to be about twice that present in the α-acyl chain. This rather random distribution of saturated and unsaturated fatty acids on the glycerol "backbone" was confirmed by the results from the pancreatic lipase hydrolysis of fraction A (Table 3).

The fatty acid analysis of fraction B by GLC of the methyl esters showed a large amount of santalbic acid (58%) and oleic acid (28%). From the intensities of the C-1 signals in the ^{13}C NMR spectrum of the TAG (Table 5), the distribution of santalbic acid appeared to be higher in the α-position than in the β-position (in a ratio of about 3:2 based on the intensity ratios of the C-1 carbon signals). This relative distribution of santalbic acid on the glycerol "backbone" was also supported by the intensities of the signals for the shifts of the carbon atoms of the enyne system, which appeared at 88.490 (C-9, α-acyl), 79.299 (C-10, α-acyl), 143.415 (C-12, α-acyl), 88.461 (C-9, β-acyl), 79.311 (C-10, β-acyl), and 143.422 (C-12, β-acyl) ppm. The lipase hydrolysis of this fraction showed a mixture of santalbic and oleic acid in the ratio of about 3:2 in the β-acyl position as the two main components.

Fraction C consisted of TAG containing santalbic acid moieties only as indicated by the ^{13}C NMR spectrum. The carbon shifts of the various carbon atoms were identical to those exhibited by the synthetic TAG of type AAA containing santalbic acid moieties (**7**) as described in Table 2. The exclusive presence of santalbic acid in this most polar fraction was further confirmed by GLC analysis of the methyl esters.

It can be concluded from these results, that ^{13}C NMR analysis of TAG fractions of seed oils provides an insight of the distribution and types of fatty acids present in the TAG mixture of a seed oil.

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Determination of Hydroperoxides and Structures by High-Performance Liquid Chromatography with Post-Column Detection with Diphenyl-1-Pyrenylphosphine

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ABSTRACT: A high-performance liquid chromatographic method, using post-column detection with diphenyl-1-pyrenylphosphine (DPPP), was developed for the quantitative and qualitative determination of isomeric lipid hydroperoxides (OOH). The OOH eluted from a normal-phase column were passed through a photodiode array detector and then mixed with DPPP solution in a reaction coil heated at 80°C. DPPP oxide formed by the reaction with OOH was determined by monitoring the fluorescence intensity at 380 nm and excitation at 352 nm. The conjugated diene OOH (13-*cis,trans*- and 9-*cis,trans*-OOH) and nonconjugated OOH (12-*cis-trans*- and 10-*cis,trans*-OOH) from photosensitized oxidation of methyl linoleate were determined in a molar ratio of 31:29:19:21, respectively. However, only the two conjugated hydroperoxides were detected by ultraviolet absorption at 234 nm. Further applications were carried out for the determination of OOH of methyl oleate and methyl linolenate. This method proved to be useful for the determination of the OOH containing both conjugated and nonconjugated diene structures.

Lipids 31, 1091–1096 (1996).

A normal-phase column high-performance liquid chromatography (HPLC) method has been applied successfully to separate positional isomers of hydroperoxides (OOH) formed as primary oxidation products of fatty acid esters (1). Ultraviolet (UV) light absorption at 234 nm due to conjugated dienes has been used to detect separated OOH isomers. However, the UV measurement at 234 nm is not specific to OOH (1,2), nor is it suitable for determination of nonconjugated OOH (3). One solution to this problem involves HPLC separation of lipid OOH with post-column chemiluminescence detection based on luminol or isoluminol oxidation during a reaction of hydroperoxides and cytochrome C (4,5). Some applications to biological systems have also been reported (6–8). Generally, nonpolar solvent systems based on *n*-hexane are used in HPLC for separation of OOH positional isomers (1). However, a post-column reaction of luminol to luminol oxide progresses only under aqueous alkaline conditions (6). Therefore, the number and type of organic solvents used for mobile phase are relatively limited, as hydrophilic nonpolar solvent mixtures do not mix well with hydrophobic luminol reagents in a post-column reaction coil.

Diphenyl-1-pyrenylphosphine (DPPP) is a recently developed fluorescent reagent which reacts specifically with various lipid OOH to form DPPP oxide even though in the presence of lipid alcohol. The DPPP oxide is excited with UV light at 352 nm and gives the emission wavelength at 380 nm (9). This reaction progresses in many different types of solvents (9), and therefore not only a reversed-phase, but also a normal-phase HPLC separation mode are useful for the DPPP fluorescence post-column detection. A few applications of this system have already been reported for analyses of OOH of triacylglycerol, cholesterol, cholesterol esters, and phospholipids in biological systems (10–12). This study was aimed at applying the DPPP fluorescence detection HPLC system to the study of lipid hydroperoxide positional isomers and their structures.

In this paper, autoxidized and photosensitized-oxidized unsaturated fatty acid methyl esters (FAME) were used as model lipids. To evaluate the efficiency of the present methology, results were compared to previous studies based on gas chromatography/mass spectrometry (GC/MS) and HPLC with UV detection.

MATERIALS AND METHODS

Lipid standard. Methyl oleate, methyl linoleate, and methyl linolenate of >99% purity were purchased from Nu-Chek-Prep. Inc. (Elysian, MN). These esters were purified by pas-

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Abbreviations: DPPP, diphenyl-1-pyrenylphosphine; FAME, fatty acid methyl ester; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; OOH, hydroperoxide; TMS, trimethylsilyl; UV, ultraviolet.

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sage through a Sep-Pak silica cartridge (Waters Associates, Milford, MA) just before use (13).

Chemicals. DPPP was kindly donated by the Tosoh Company (Tokyo, Japan). Methylene blue trihydrate and $NaBH_4$ (Wako Pure Chemicals, Osaka, Japan) were of reagent grade. *n*-Hexane of HPLC grade was used after glass-distillation. Diethyl ether of reagent grade was purified by a combination of the first washing with ferrous sulfate–sulfuric acid solution to remove peroxide impurities followed by distillation in glass. Methanol, 1-butanol, and acetone (all HPLC grade) were used as purchased.

Autoxidation of lipids. One hundred mg of FAME were dissolved in *n*-hexane and placed into a glass test tube (10 mm × 13 mm). *n*-Hexane was flushed out with a nitrogen stream. Autoxidation of FAME was carried out in air at 35°C in the dark. The autoxidized FAME was dissolved in *n*-hexane and separated by HPLC.

Photosensitized oxidation of lipids. The oxidation of FAME was sensitized with methylene blue (1 mg/g ester) in a methanol solution with continuous gentle stirring in a jacketed flask cooled to 3°C. The solution in the flask was exposed to a 1000-watt tungsten light source through a 3-cm layer of water to filter out infrared radiation (14). Methanol was evaporated under a nitrogen stream in the dark and the residual photosensitized-oxidized lipid was dissolved in 25% diethyl ether in *n*-hexane. The solution was passed through a Sep-Pak silica cartridge to remove the photosensitizer. The eluent was made up to 10 mL with *n*-hexane and 20 µL solution was injected to the HPLC column.

The HPLC system. A schematic of the HPLC system used is shown in Figure 1. The mobile phase passed through an HPLC column with an HPLC pump (model 1050; Hewlett-Packard, Boise, ID). The eluant from the column was then passed through a diode array detector (HP 1040A) and subsequently to a stainless-steel reaction coil (0.5 mm i.d. × 10 m). At the inlet of the reaction coil, a DPPP reagent solution was pumped (HPLC pump, model 45; Waters Associates) and mixed with the eluent in a T-connector. The reaction coil was immersed in a water bath heated at 80°C. The eluent from the coil was cooled to room temperature by passing through a stainless steel coil (0.5 mm i.d. × 5 m), immersed in a water tank, and led to a fluorescence photospectrometer (model 650-40; Perkin Elmer, Norwark, CO). The intensity of the fluorescence signal was integrated with a chromatographic integrator (Hewlett-Packard model 3390A).

Separation and detection of OOH. A normal-phase column (Supelcosil LC-Si, 2.1 mm i.d. × 250 mm, 5 µm; Supelco, Bellefonte, PA) was used to separate positional isomers of fatty ester OOH. A mixture of 500 mL of *n*-hexane and 34 mL of diethyl ether was pumped at a rate of 0.6 mL/min. The DPPP solution (3 mg in a mixture of 200 mL 1-butanol and 200 mL methanol) was pumped at a flow rate of 0.6 mL/min.

Lipid OOH eluted from the column were passed through the dioide array detector and the absorption due to conjugated dienes was monitored at 234 nm (1). The fluorescence intensity of the DPPP oxides formed by reacting lipid hydroperoxides and DPPP was monitored at 380 nm with excitation at 352 nm (9).

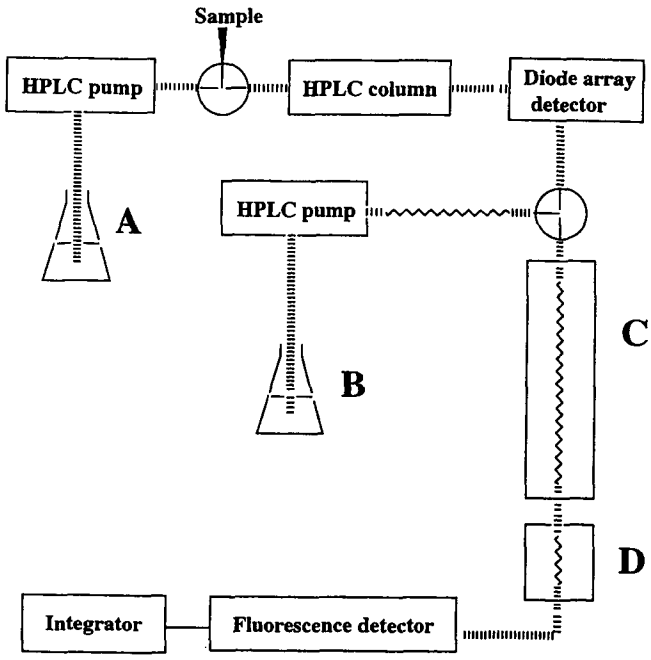


FIG. 1. Diagram of the diphenyl-1-pyrenylphosphine (DPPP)-fluorescence detection high-performance liquid chromatography (HPLC) system. A, HPLC pump for a mobile phase; B, HPLC pump for the DPPP solution; C, reaction coil; D, cooling coil.

GC/MS. OOH positional isomers separated by the HPLC column and monitored with the diode array detector were fractionated before mixing with the DPPP solution by disconnecting the flow tubing at the inlet of the T-connector behind the column. Butylated hydroxyanisole (200 ppm) was added to suppress formation of secondary oxidation products. The hydroperoxides were reduced to the corresponding hydroxy esters with $NaBH_4$ (15), followed by hydrogenation to avoid rearrangement of hydroperoxides, and subsequently converted to trimethylsilyl (TMS) ether derivatives by adding hexamethyldisilazane and trimethylchlorosilane (14).

The TMS derivatives were separated with a gas chromatography (model GC 17A; Shimadzu, Kyoto, Japan) equipped with a Supelcowax-10 fused silica open-tubular column (0.25 mm i.d. × 25 m, 0.25 µm; Supelco Japan Ltd., Tokyo, Japan).

The outlet of the column was connected directly to an electron impact ion source of a quadrupole mass spectrometer (Shimadzu model QP 5000). The column oven temperature was programmed from 150 to 220°C at 1°C/min. Injection port temperature was 250°C. Helium was used as carrier gas. Mass spectra were acquired using 3 KV accelerating energy, 70 eV electron beam energy, and a source temperature of 260°C by using a computer system (Shimadzu Class-5000).

RESULTS

Separation of positional isomers of methyl linoleate OOH. Photosensitized-oxidized methyl linoleate showed four peaks

by HPLC with post-column DPPP fluorescence detection (Fig. 2A). However, only two peaks were shown by UV at 234 nm (Fig. 2B). MS (data not shown) confirmed the identity of the positional isomers as follows: 13-*cis,trans*-, 12-*cis,trans*-, 9-*cis,trans*-, and 10-*cis,trans*-hydroperoxy methyl linoleates based on the literatures (15,16).

Typical chromatograms of autoxidized methyl linoleate OOH detected by fluorescence at 380 nm and absorption at 234 nm (Fig. 2, C and D). Four peaks, including methyl linoleate 13-*cis,trans*-, 13-*trans,trans*-, 9-*cis,trans*-, and 9-*trans,trans*-hydroperoxy isomers, were identified by MS of the corresponding TMS derivatives of hydroperoxides separated by HPLC (data not shown).

Separation of other fatty acid methyl ester OOH. Peak assignment was confirmed for every peak component fractionated by HPLC (data not shown). Although methyl 10-*cis,trans*- and 9-*cis,trans*-hydroperoxy monoene hydroperoxides could not be detected by UV absorption at 234 nm (Fig. 3B), the isomers were detected by DPPP-fluorescence (Fig. 3A). For photosensitized-oxidized methyl linolenate, the peaks due to 13-*cis,trans*-, partially separated 16-*cis,trans*- and 12-*cis,trans*-, 15-*cis,trans*-, 9-*cis,trans*-, and 10-*cis,trans*-methyl linolenate OOH were readily measured by DPPP-fluorescence detection (Fig. 3C). The nonconjugated hydroperoxide isomers, 15-*cis,trans*- and 10-*cis,trans*-methyl linolenate hydroperoxy, were not detected by UV absorption at 234 nm (Fig. 3D).

Quantitative analyses. Tables 1, 2, and 3 summarize peak area ratios of OOH of methyl oleate, linoleate, and linolenate photosensitized-oxidized to different peroxide values. Previously reported results show that the ratios of isomeric OOH formed from photosensitized-oxidized methyl esters were 47.7–51:52.3–49 for methyl oleate (14, 15,17). For 13-*cis,trans*-, 12-*cis,trans*-, 9-*cis,trans*-, and 10-*cis,trans*-hydroperoxides, the previously reported ratios were 34.5–35.0, 17.0–17.0, 31.9–32.0, 16.7–17.0 (14,15).

Reproducibility of the determination of hydroperoxides by this HPLC system was evaluated (Table 4). For the hydroperoxides from methyl oleate and from methyl linoleate, good reproducibility in determination of peak areas was obtained. However, for the methyl linolenate OOH, the peak areas of 10-*cis,trans*-hydroperoxy methyl linolenate were not reproducible due to miss integration of peak area. The use of peak height for quantification may resolve this problem.

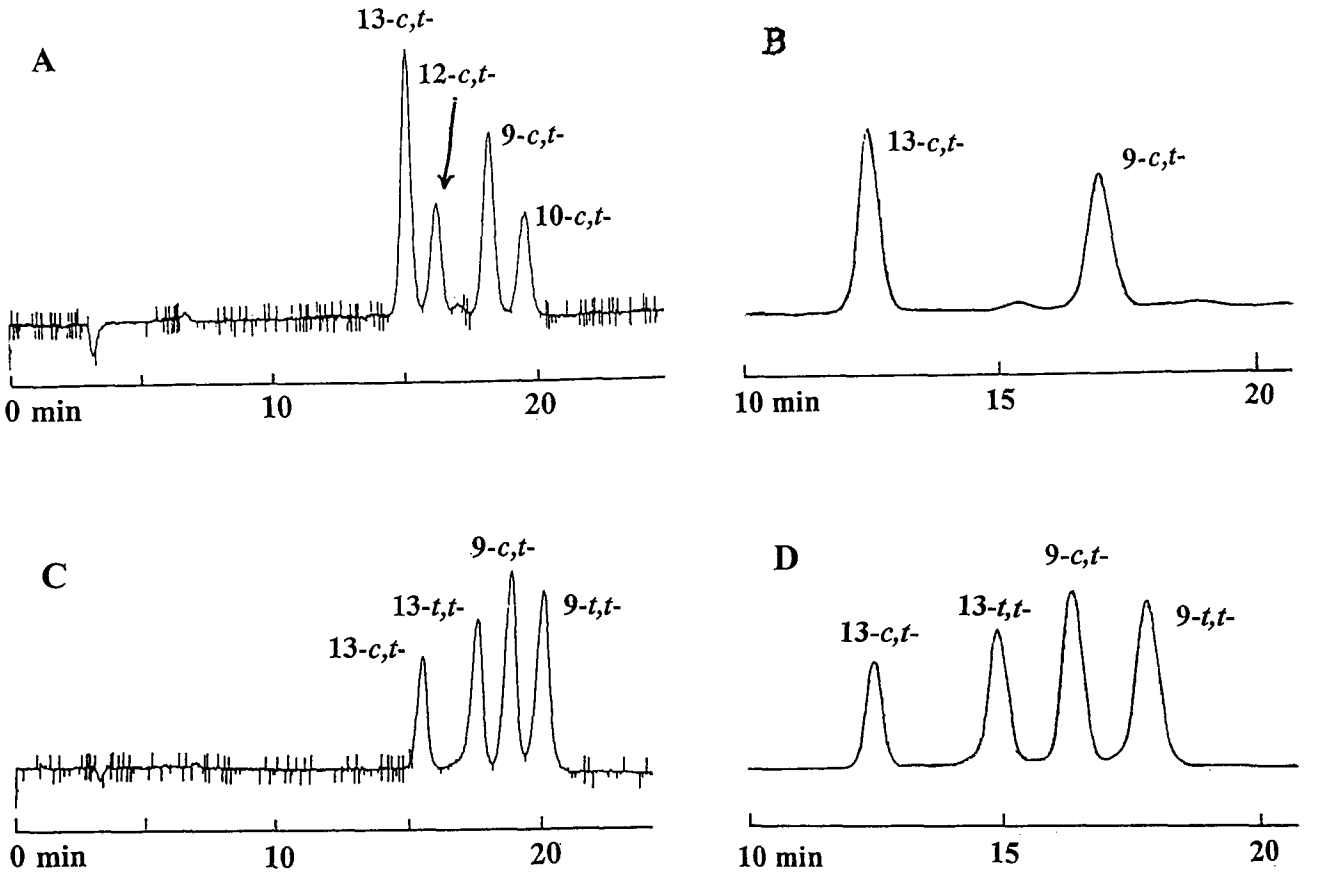


FIG. 2. Typical chromatograms of methyl linoleate isomeric hydroperoxides. Photosensitized-oxidized methyl linoleate detected by the DPPP-fluorescence detection (A) and by ultraviolet (UV) absorption at 234 nm (B). Autoxidized methyl linoleate detected by the DPPP-fluorescence (C) and by UV light absorption at 234 nm (D). See Figure 1 for abbreviations; *c,t-, cis,trans-*.

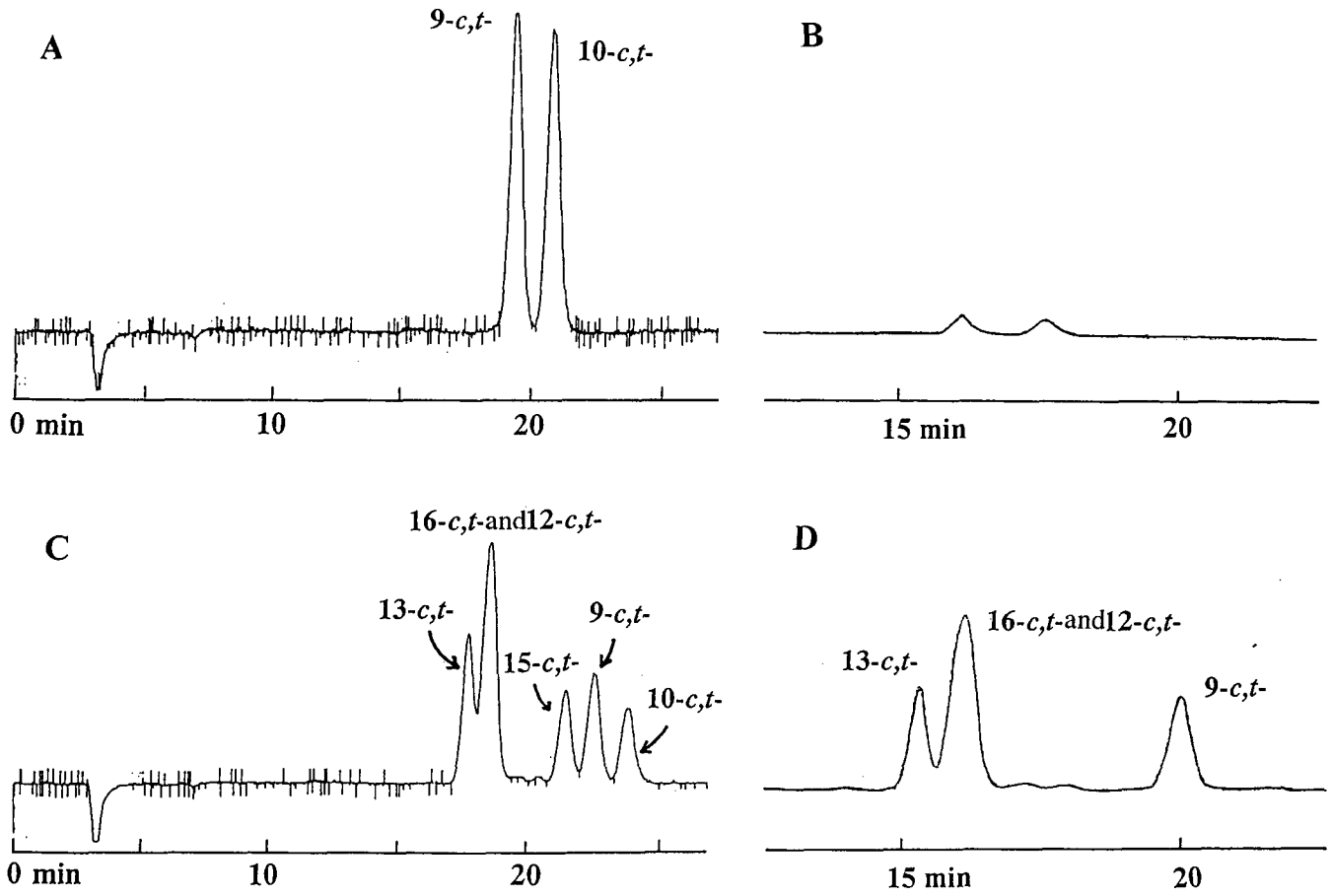


FIG. 3. Typical chromatograms of methyl oleate and methyl linolenate isomeric hydroperoxides. A and C, photosensitized-oxidized methyl oleate and methyl linolenate hydroperoxides, respectively, detected by DPPP-fluorescence; B and D, detected by absorption at 234 nm. See Figures 1 and 2 for abbreviations.

DISCUSSION

This study showed that the DPPP fluorescence detection coupled with HPLC is a simple and accurate method to determine the distribution of OOH isomers of methyl oleate, linoleate, and linolenate. Autoxidation of methyl linoleate produced four conjugated positional isomers of monohydroperoxides, i.e., 13-*cis,trans*-OOH, 13-*trans,trans*-OOH, 9-*cis,trans*-OOH, and 9-*trans,trans*-OOH, which can be analyzed by UV light absorption (1).

However, the nonconjugated singlet oxygen oxidation products of methyl linoleate, 12-*cis,trans*-OOH and 10-*cis,trans*-OOH, are not detectable by UV light absorption. These nonconjugated hydroperoxides are detectable by the DPPP fluorescence detection system, because DPPP is directly oxidized by hydroperoxides to form a fluorescent DPPP oxide. However, in the chemiluminescence detection, several kinds of oxygen radicals and excited oxygens oxidize luminol (5). In the chemiluminescence detection, nonconjugated OOH isomers of methyl linoleate and methyl linolenate were detectable in HPLC, however, separation between certain isomers was not completed (18). Nonlipid components such as sugar, amino acids and ascorbic acid contaminated into a methanol-aqueous layer in a stage of lipid extraction interfere with the accurate analysis of hydroperoxides by causing quenching of the chemiluminescence (5).

It is well accepted that oxidation by singlet oxygen takes place at both ends of the C=C bond (17). Therefore, photosensitized oxidation of methyl oleate forms only two isomers, i.e., 9-*cis,trans*-OOH and 10-*cis,trans*-OOH (13–15,17). The ratios of 9-*cis,trans*-OOH to 10-*cis,trans*-OOH reported little variation; 50:50 by GC/MS (17), 47.7:52.3 by GC/MS (14), and 51:49 by HPLC (13).

A number of previous studies on measurement of lipid hydroperoxide have been carried out by GC/MS. Derivatizations of OOH, including reduction and hydrogenation of hydroperoxy group to the corresponding hydroxy group and subsequent conversion to TMS ether group, are required before GC/MS analysis (14,19–21). These derivatizations are usually time-consuming and can possibly form artifacts during treatment of a sample OOH as well as unsaturated lipids. In contrast, the DPPP fluorescence detection system requires no derivatization of the sample.

It was also possible to determine monohydroperoxides at

TABLE 1
Relative Ratios of Photosensitized-Oxidized Methyl Oleate Hydroperoxide Positional Isomers (%)

Method	PV[a]	9-*c,t*-[b]	10-*c,t*-[b]	References
HPLC/DPPP[c]	36	50.0	50.0	
	38	47.5	52.5	
	92	51.3	48.7	
	98	50.3	49.7	
	171	50.5	49.5	
	179	52.0	48.0	
HPLC[d]	1727	47.7	52.3	13
GC/MS[e]	1727	51	49	14
GC/MS[e]	—	50	50	17

[a]Peroxide value (meq/kg).
[b]*c,t-, cis,trans-*.
[c]DPPP, diphenyl-1-pyrenylphosphine.
[d]Separated corresponding hydroxystearate and detected by ultraviolet at 212 nm. HPLC, high-performance liquid chromatography.
[e]Analyzed corresponding trimethylsilylated hydroxystearate. GC/MS, gas chromatography/mass spectrometry.

only picomole levels by this DPPP system. High specificity and sensitivity to hydroperoxides may be useful for kinetic studies on oxidation of polyunsaturated fatty acids in a biological membrane systems including effects of antioxidant compounds.

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TABLE 2
Relative Ratios of Photosensitized-Oxidized Methyl Linoleate Hydroperoxide Positional Isomers (%)[a]

Method	PV	13-*c,t*-	12-*c,t*-	9-*c,t*,-	10-*c,t*,-	References
HPLC/DPPP	106	36.2	15.1	33.3	15.5	
	107	34.2	12.8	36.0	17.0	
	163	33.5	17.5	31.5	17.6	
	164	30.3	16.6	33.5	19.5	
	283	30.5	18.5	30.7	20.2	
	274	29.2	17.8	32.4	20.8	
	357	30.8	19.3	29.5	20.4	
	340	29.1	18.3	31.2	21.4	
HPLC[b]	1124	35	17	32	17	13
HPLC[c]	—	31.1	20.7	33.3	14.7	18
GC/MS[d]	1124	34.5	17.0	31.9	16.7	14

[a]See Table 1 for abbreviations.
[b]Separated corresponding hydroxystearate and detected by ultraviolet at 212 nm.
[c]Separated corresponding hydroperoxide and detected by chemiluminescence.
[d]Analyzed corresponding trimethylsilylated hydroxystearate.

TABLE 3
Relative Ratios of Photosensitized-Oxidized Methyl Linolenate Hydroperoxide Positional Isomers (%)[a]

Methods	PV	13-*c,t*-	16-*c,t*- and 12-*c,t*-	15-*c,t*-	9-*c,t*-	10-*c,t*-	Refs.
HPLC/DPPP	78	17.7	41.7	8.2	18.2	14.2	
	85	18.5	40.2	11.6	16.1	13.5	
	284	18.2	38.4	13.4	17.0	13.0	
	286	18.3	39.4	13.2	16.3	13.9	
	634	17.5	38.5	13.8	16.9	13.3	
	644	17.5	38.9	13.5	17.8	12.3	
HPLC[b]	1566	15	40	12	20	13	13
GC/MS[c]	1566	14.0	37.2	13.4	22.7	12.7	14

[a]See Table 1 for abbreviations.
[b]Separated corresponding hydroxystearate and detected by ultraviolet at 212 nm.
[c]Analyzed corresponding trimethylsilylated hydroxystearate.

TABLE 4
Standard Deviations of the Isomeric Hydroperoxide Analyses by HPLC/DPPP[a]

Isomeric hydroperoxides	Relative ratio (%)[b]	SD[c]
Methyl oleate/OOH, PV = 92		
9-*c,t*-	50.5 ± 0.89	2.1
10,*c,t*-	49.5 ± 0.89	
Methyl linoleate-OOH, PV = 123		
13-*c,t*-	30.8 ± 0.70	0.5
12-*c,t*-	19.4 ± 0.18	
9-*c,t*-	29.3 ± 0.59	
10-*c,t*-	20.6 ± 0.26	

[a]See Tables 1 and 3 for abbreviations. OOH, hydroperoxide.
[b]Mean ± SD (n = 5).
[c]Standard deviation on HPLC by analysis of variation (Ref. 13).

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Difficulties in the Assay of Phosphatidate Phosphohydrolase Activity. Influence of Ionic Strength, Detergent, and Selection of Substrate

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ABSTRACT: In the present paper, problems in connection with assay of the activity of magnesium-dependent rat liver phosphatidate phosphohydrolase (PAP) are discussed. PAP activity is usually measured by following the production of diacylglycerol or inorganic phosphate from the substrate phosphatidate. These two methods may give widely different results due to a number of factors that may affect the assay. One such factor is the composition of the substrate. Higher apparent enzyme activity was observed with dioleoyl-phosphatidate than with dipalmitoyl-phosphatidate. This substrate-dependent difference in apparent PAP activity was 2–2.5-fold in the absence and 10-fold in the presence of Triton X-100, respectively. Triton X-100 reduced the activity as measured with the dipalmitoyl-phosphatidate substrate. In contrast, the activity of PAP as measured with dioleoyl-phosphatidate was stimulated by Triton X-100. The stimulatory effect of Triton was reduced or abolished when the ionic strength in the assay mixture was increased. Assays based on ^{32}P-labeled substrate are rapid and sensitive. It is shown here that ^{33}P can be used as an alternative. This radionuclide has a longer half-life and also emits particles with lower energy, thus posing less potential health hazards for the user.
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Phosphatidate phosphohydrolase (PAP; EC 3.1.3.4.) catalyzes the dephosphorylation of phosphatidate, producing diacylglycerol and inorganic phosphate. This reaction occurs at an important branch-point in glycerolipid metabolism. Diacylglycerol is the immediate precursor for triacylglycerol and is also used for the synthesis of the major phospholipids phosphatidylcholine and phosphatidylethanolamine. PAP is considered to play an important role in controlling the rate of synthesis of liver glycerolipids, in particular that of triacylglycerols (1–3). Two forms of PAP activity that differ from each other in various respects have been identified (4,5). One form, the activity of which is independent of magnesium ions and insensitive to inhibition by *N*-ethylmaleimide (NEM), is present predominantly in the plasma membrane. This enzyme is thought to play a role in cell-signalling by changing the local concentrations of the second messengers phosphatidate and diacylglycerol. The other enzyme form, present in cytosolic and microsomal fractions, is magnesium-dependent and NEM-sensitive and is regarded as a metabolic form, operative and with regulatory functions in glycerolipid synthesis.

PAP activity is generally measured by following the formation of diacylglycerol or the release of inorganic phosphate. It is often difficult to compare results obtained by different assay protocols since the enzyme activity could be affected by a number of factors, such as the composition of the phosphatidate substrate, presence of detergents, etc. In the present paper, attempts are made to define some of these different factors and evaluate their importance for the apparent enzyme activity. Furthermore, the use of ^{33}P as an alternative to ^{32}P in the phosphate determination assay method is described.

MATERIALS AND METHODS

Enzyme preparation. Microsomal and cytosolic fractions were prepared from the livers of male 200 g Sprague-Dawley rats fed ordinary laboratory chow *ad libitum* (6). PAP was purified from the cytosolic fractions by hydroxylapatite chromatography (7). In order to induce PAP activity, the rats received an intraperitoneal injection of ethanol 16–18 h before they were killed (7–9).

Preparation of radioactively labeled substrates. ^{32}P-labeled phosphatidate was prepared by incubation of γ-^{32}P-ATP (DuPont NEN, Boston, MA) and diolein with *Escherichia coli* diacylglycerol kinase (Calbiochem, La Jolla, CA) as described by Day and Yeaman (5). ^{33}P-phosphatidate was prepared from diolein and γ-^{33}P-ATP (DuPont NEN) using the same procedure. Phosphatidate was separated from water-soluble substances by extraction and was used in the assay without further purification (as described in Ref. 5). The amount of diolein carried over with the nonpurified radioactive substrate would give a diolein concentration of about 10 μM in

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Abbreviations: NEM, *N*-ethylmaleimide; PAP, phosphatidate phosphohydrolase.

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the assay. Addition of diolein to the purified (thin-layer chromatography, see below) and nonpurified substrate in amounts corresponding to 10 μM (and even 50 μM) final concentration of diolein in the assay mixture did not affect the enzyme activity measured in the absence and in the presence of Triton X-100 (Sigma Chemical Corporation, St. Louis, MO) using the assay system described below. The enzyme activity measured in control experiments with purified radioactively labeled dioleoyl-phosphatidate substrate in the absence and in the presence of Triton X-100 and at low and high (up to 200 mM) concentrations of potassium phosphate was the same as with the nonpurified radioactive substrate. Dioleoyl-phosphatidate was stored in chloroform solution at –20°C under argon. Prior to use, nonradioactive phosphatidate (L-α-phosphatidic acid, dioleoyl; Sigma) was added to the radioactive phosphatidate to a specific radioactivity of 1000–2000 cpm/nmol, the solvent was evaporated under a stream of argon, and 10 mM Tris/HCl buffer, pH 7.4, containing 3 mM EDTA and 4 mM EGTA was added. The phosphatidate was either sonicated for one min in 15-s bursts or solubilized by the addition of Triton X-100. Sonicated phosphatidate was slightly turbid while the addition of the detergent produced a clear solution.

In some experiments ^{32}P- or ^{33}P-labeled phosphatidate was prepared from dipalmitin instead of diolein. This phosphatidate substrate was purified before use (thin-layer chromatography, same as for [^{14}C]phosphatidate, see below), since the incorporation of phosphate into dipalmitin was much lower than into diolein. The radioactive dipalmitoyl-phosphatidate was stored in chloroform/methanol (2:1) at –20°C under argon. Prior to use, the solvent was evaporated, and nonradioactive phosphatidate (L-α-phosphatidate, dipalmitoyl; Sigma), dispersed by sonication in 10 mM Tris/HCl buffer, pH 7.4, was added. Labeled and unlabeled phosphatidate was then sonicated together or solubilized with Triton as described above. In order to obtain a clear solution, the dipalmitoyl-phosphatidate/Triton mixture had to be heated (2–3 min at 55°C). When cooled to room temperature, this solution was still clear.

[^{14}C]phosphatidate was prepared by incubation of rat liver microsomes with glycerol-3-phosphate and [1-^{14}C]palmitic acid or [1-^{14}C]oleic acid, extracted with chloroform and purified by thin-layer chromatography (10).

Enzyme assay. (i) Phosphate determination method. Unless otherwise specified PAP activity was measured for 10 min at 37°C by following the release of water-soluble ^{32}P or ^{33}P from 0.5 mM ^{32}P- or ^{33}P-labeled phosphatidate in 50 mM Tris/HCl buffer, pH 7.0, containing 2.5 mM $MgCl_2$, 0.2 mg bovine serum albumin (fatty acid free, Sigma) per mL, 1 mM EDTA, 1 mM EGTA and 1 mM dithiothreitol, in the presence or absence of 5 mM Triton X-100.

When Triton X-100 was present, it was mixed with the phosphatidate substrate at a molar ratio of 10:1 before it was added to the assay system. In control experiments with microsomal and cytosolic (crude and purified by hydroxylapatite chromatography) PAP, the concentration of Triton X-100 was varied from 2.5 mM to 15 mM (in 2.5 mM steps) at 0.5 mM dioleoyl-phosphatidate and 0–2.5 mM potassium phosphate in order to ensure that 10:1 was an optimal detergent:phosphatidate ratio.

Total volume of the incubation mixture was 0.1 mL. The reaction was terminated by the addition of 0.5 mL of 0.1 M HCl in methanol. Chloroform (1 mL) and 1 M $MgCl_2$ (1 mL) were then added. After thorough mixing, the phases were allowed to separate and a 1 mL aliquot of the aqueous layer was transferred to a scintillation vial and radioactivity determined in 8 mL of Lumasafe (LUMAC LSC B.V., Groningen, The Netherlands), using a liquid scintillation counter (1217 Rackbeta; LKB-WALLAC, Stockholm, Sweden).

(ii) Diacylglycerol determination method. Unless otherwise specified, PAP activity was measured in 0.8 mL incubation mixtures for 10 or 15 min at 37°C by following the production of [^{14}C]diacylglycerol from (a) 0.625 mM [^{14}C]phosphatidate in 75 mM Tris/maleate buffer, pH 6.9, containing 2 mM $MgCl_2$, 0.25 mM EDTA, and 0.05–0.1 mM dithiothreitol or (b) 0.5 mM [^{14}C]phosphatidate in 50 mM Tris/HCl buffer, pH 7.0, containing the same additives as described above for the phosphate determination method. The reaction was terminated by the addition of five volumes of chloroform/methanol (2:1, vol/vol). In order to identify the reaction products, the chloroform phase was subjected to thin-layer chromatography, and the chromatoplates were scanned for radioactivity using a computerized radioscanner (for more details see Refs. 7,10).

(iii) Treatment with NEM. In experiments performed to test the effect of NEM on PAP activity, the samples were preincubated for 10 min at 37°C with or without 4.2 mM NEM prior to assay.

RESULTS AND DISCUSSION

For several years, the diacylglycerol detection method, a in the Materials and Methods section under paragraph (ii), has been our standard method for measuring magnesium-dependent NEM-sensitive PAP activity in rat and other mammalian tissues. By the convenient technique with radioscanning of the chromatoplates, diacylglycerol is identified and the relative amounts of substrate remaining and product formed are calculated. This method also offers the possibility to detect other labeled products that might be formed as the result of other contaminating enzyme activities.

Although giving reproducible results, the diacylglycerol determination method bears the disadvantages of being time-consuming and not sufficiently sensitive for all purposes. In some experiments we have, therefore, chosen to assay PAP activity using the faster and more sensitive method with determination of ^{32}P-phosphate released.

As discussed in e.g., References 11–13, interference by other enzyme activities present in different amounts in different tissues and cellular fractions can create problems. Thus, the presence of diacylglycerol lipase would underestimate PAP activity determined by measuring the formation of dia-

cylglycerol. Lipase activity can be inhibited by diisopropylfluorophosphate (4,12), tetrahydrolipstatin (4), and, at least partially, by phenylmethylsulfonyl fluoride (14). When the activity of lipase is high, it might be more convenient to assay PAP activity by measuring the release of water-soluble phosphate. On the other hand, this latter method can be invalid in the presence of phospholipases, degrading phosphatidate to glycerol-3-phosphate (that also can be further degraded by various phosphatase activities), thus leading to overestimation of the activity of PAP. Consequently, it is advantageous to include both diacylglycerol and phosphate determination techniques in the methodological arsenal, at least when working with PAP in crude systems.

^{33}P as an alternative to ^{32}P. The short half-life (14.3 d) and the high energy of the radiation emitted (maximum of 1.709 MeV) from the ^{32}P isotope can be considered disadvantageous in many cases. Recently, ^{33}P-ATP has been introduced as an alternative to ^{32}P-ATP. This radionuclide (i.e., ^{33}P) has a longer half-life (25.4 d) and emits particles with much lower energy (maximum 0.249 MeV). Thus, the PAP substrate with radioactive phosphate does not have to be prepared as often using ^{33}P compared to ^{32}P, and, both as a result of this, and because of the shorter range of ^{33}P-radiation, the hazard for the experimentator is reduced. We have prepared ^{33}P-phosphatidate and found the results when testing PAP activity with this substrate to be comparable to those obtained with ^{32}P-phosphatidate. Due to the low energy of the radiation, ^{33}P has to be counted in a scintillator. A ^{32}P-channel can be used, as proposed by NEN (^{33}P-Nucleotide Applications Guide obtained from NEN), but the energy spectrum of the radiation more closely resembles that of ^{14}C. Using a ^{14}C- or a ^{33}P-channel gives higher readings and should be preferred. Alternatively, as also proposed by NEN, a wide open channel can be used. Comparing the readings obtained in different channels, we found that the relative radioactivity of γ-^{33}P-ATP samples was 100% in the wide open channel, 98% in the ^{33}P- and ^{14}C-channels, and 61% in the ^{32}P-channel, respectively.

Phosphatidate species. Differences in apparent PAP activity observed when comparing results obtained with the two assay methods (i.e., determination of diacylglycerol or phosphate) may be due to differences in the composition of the assay mixture. When PAP activity is measured as formation of labeled diacylglycerol, the assay generally involves use of a phosphatidate substrate with palmitoyl acyl chains. In contrast, release of inorganic ^{32}P-phosphate is usually measured with a substrate containing oleoyl acyl chains. In the former case, in which the substrate is labeled with the long-lived ^{14}C(or ^{3}H)-isotope, it seems practical to use chemically stable saturated acyl chains. In addition, [1-^{14}C]palmitic acid that is used for preparation of the substrate is less expensive than [1-^{14}C]oleic acid. When the release of inorganic radiolabeled phosphate is measured, an isotope with a short half-life is used, and thus there is less need for long chemical stability. In addition, at least in our hands, the diolein used for preparation of the substrate, and the unlabeled dioleoyl-containing phosphatidate used to dilute the radioactive substrate are more easily dispersed than the corresponding palmitoyl species. Furthermore, the activities of some isozymes of PAP have been reported to be higher when measured by determination of phosphate released from dioleoyl-phosphatidate as compared to dipalmitoyl-phosphatidate (e.g., 15–18). Confirming this, using both the diacylglycerol- and the phosphate determination method, we have found the activity of rat liver cytosolic PAP, purified by hydroxylapatite chromatography, to be 2–2.5-fold higher with dioleoyl-phosphatidate than with the dipalmitoyl substrate. In contrast, with membrane-bound substrate, the soluble rat liver PAP has been reported to display higher activity with dipalmitoyl- than with dioleoyl-phosphatidate (19). This discrepancy might result from differences in the physical states of membrane-bound and sonicated/solubilized substrate or from interference by some component(s) in the membranes.

There was a good correlation between the diacylglycerol- and phosphate-determination methods as exemplified by the experiment on rat liver cytosolic PAP illustrated in Figure 1 and also when testing the effect of pravastatin treatment on the activity of PAP in rat liver microsomes. Treatment of rats with pravastatin has recently been shown to increase the activity of liver PAP, as measured by the diacylglycerol determination method (a in the Materials and Methods section) (6), and this increase was found to be of the same magnitude when the activity was assayed by measuring the release of phosphate (not shown).

Influence of Triton X-100 and ionic strength. Some investigators measure PAP activity in the presence of Triton X-100. The detergent is often added, together with the substrate, in a mixed micelle at a molar ratio of Triton X-100 to phosphati-

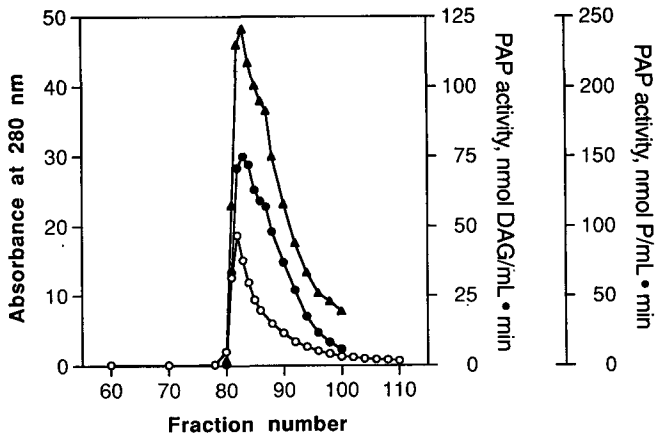


FIG. 1. Hydroxylapatite chromatography of rat liver cytosol as described in Ref. 7. The enzyme was eluted with 400 mM potassium phosphate, pH 7.4, containing 30% (vol/vol) glycerol, 1 mM EDTA, and 0.1 mM dithiothreitol. Absorbance at 280 nm (○); activity measured as ^{14}C-diacylglycerol (DAG) formed from [^{14}C]dipalmitoyl-phosphatidate (●) and as water-soluble ^{33}P-phosphate (P) released from ^{33}P-labeled dioleoyl-phosphatidate (▲). In both cases the assay mixture contained 0.625 mM phosphatidate, 75 mM Tris/maleate buffer, pH 6.9, 2 mM $MgCl_2$, and 0.25 mM EDTA. Triton was not present; PAP, phosphatidate phosphohydrolase.

date of 10:1 (e.g., 5,20). The NEM-sensitive, magnesium-dependent and the NEM-insensitive, magnesium-independent forms of rat liver PAP are reported to be differently affected by Triton X-100. The activity of the NEM-insensitive PAP in crude subcellular fractions is stimulated by Triton X-100 (4,5). The activity of the NEM-sensitive form has been reported to be stimulated (although to a lesser degree) by Triton when assayed in the absence of phosphatidylcholine (5). However, in the presence of phosphatidylcholine, Triton has an inhibitory effect on the activity (4).

We have earlier reported that partially purified magnesium-dependent rat liver PAP assayed in the absence of phosphatidylcholine and with [^{14}C]dipalmitoyl-phosphatidate as the substrate is inhibited by several detergents (octylglucoside, sodium cholate, and Tween 20; Ref. 7). In the present investigation, we have found that the enzyme is inhibited also in the presence of Triton X-100 when assayed with dipalmitoyl-phosphatidate as the substrate using either the phosphate- or the diacylglycerol determination method. The inhibitory effect was observed irrespective of whether the enzyme was assayed at low concentration of potassium phosphate (2.5 mM; Fig. 2) or at higher (100 mM; not shown) concentration of potassium phosphate and irrespective of whether the dipalmitoyl-phosphatidate/Triton substrate used was slightly turbid or clarified by warming (as described in the Materials and Methods section).

However, when assayed with dioleoyl-phosphatidate as the substrate, the enzyme activity was stimulated in the presence of 5 mM Triton X-100, provided that the concentration of potassium phosphate in the assay was not too high (Fig. 3A). Also the presence of higher concentrations of Tris/HCl buffer reduced the stimulation obtained by Triton. The stimulatory effect of 5 mM Triton on the activity of the purified enzyme was not due to the presence of magnesium-

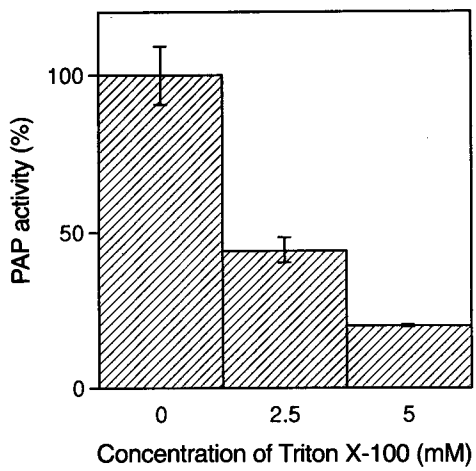


FIG. 2. Activity in the absence and in the presence of Triton X-100 of rat liver cytosolic PAP purified by hydroxylapatite chromatography. The figure shows mean and range of triplicate determinations of the activity using the phosphate determination assay method with ^{33}P-dipalmitoyl-phosphatidate as the substrate. The final concentration of potassium phosphate in the assay was 2.5 mM. See Figure 1 for abbreviation.

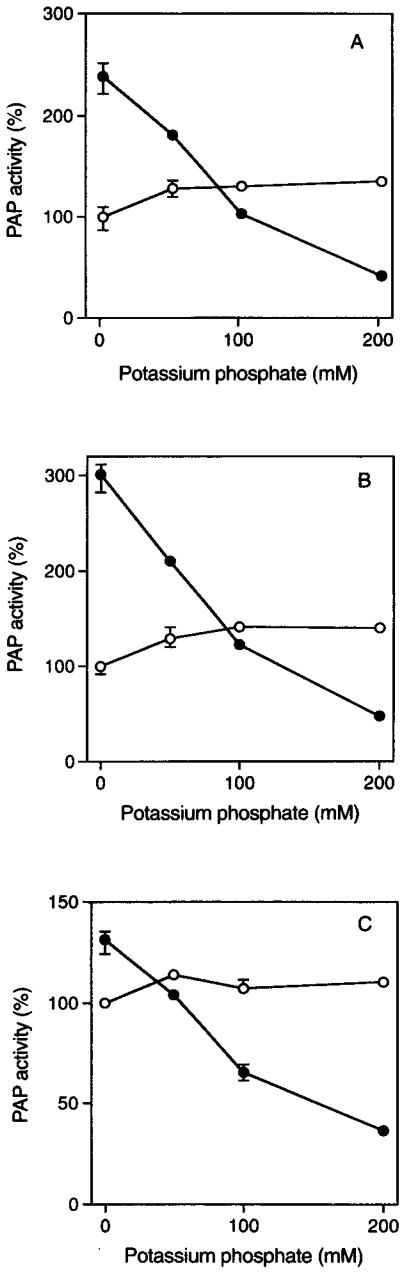


FIG. 3. Effect of increasing concentrations of potassium phosphate on PAP activity. Microsomal and cytosolic (crude and purified by hydroxylapatite chromatography) PAP activity in the absence (○) and in the presence (●) of 5 mM Triton X-100. The figure shows mean and range of triplicate determinations of the activity using the phosphate determination assay method with ^{33}P-dioleoyl-phosphatidate as the substrate. Where no error bars are shown, the variation was small enough to be contained within the area of the symbol. The enzyme sample constituted one-fourth of the total incubation volume. Prior to assay, the microsomal and cytosolic fractions were diluted with the buffer used for homogenization of the livers (i.e., 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA, and 0.2 mM dithiothreitol), and the purified enzyme (OH-ap pool) was diluted with 10 mM potassium phosphate buffer, pH 7.4, containing 30% (vol/vol) glycerol, 1 mM EDTA, and 0.2 mM dithiothreitol. The potassium phosphate concentration (final concentrations in the assay are given) was adjusted by adding potassium phosphate buffer, pH 7.0, to the assay mixtures. A: OH ap pool; B: cytosol; C: microsomes. See Figure 1 for abbreviation.

independent PAP since no activity could be detected in the absence of magnesium. Concentrations of Triton higher than 5 mM were inhibitory. For comparison, the effect of 5 mM Triton on the activity of PAP in the cytosolic and microsomal fractions of rat liver is also shown (Fig. 3B and C). PAP activity in the cytosolic fraction was also very low in the absence of magnesium (not exceeding 5% of the activity in the presence of magnesium). The contribution of the NEM-insensitive, magnesium-independent PAP activity to the total microsomal PAP active on dioleoyl-phosphatidate in the absence of potassium phosphate and in the presence of 100 mM potassium phosphate was estimated by analyzing samples preincubated with or without NEM. It was found that the NEM-insensitive activity amounted to about 10% of the total activity in the absence and about 20% in the presence of Triton X-100.

Increasing concentrations of NaCl and KCl produced stimulation and inhibition of the enzyme activity similar to but not as pronounced as that shown in Figure 3 for potassium phosphate. Sodium phosphate (200 mM) was as effective in these respects as potassium phosphate (200 mM). Exchanging 10 mM Tris/HCl for 10 mM potassium phosphate or 0.25 M sucrose for 30% glycerol in the buffer used for dilution of the enzyme prior to assay did not affect the results obtained with low and high (100 mM) potassium phosphate (other salts and concentrations were not tested in this respect).

Concluding remarks. Thus, in conclusion, when assayed in the presence of Triton X-100, the apparent activity of the magnesium-dependent rat liver PAP differed up to at least tenfold, depending on whether dioleoyl- or dipalmitoyl-phosphatidate was used as the substrate. The enzyme showed higher activity when assayed with dioleoyl-phosphatidate than with dipalmitoyl-phosphatidate. Triton X-100 stimulated the former activity and inhibited the latter activity. The stimulatory effect of Triton was, however, reduced or abolished when the ionic strength in the assay mixture was increased. An interpretation of the stimulating effect of Triton X-100 might be that the dioleoyl-phosphatidate/Triton X-100 mixed micelle mimics the natural substrate more closely than does dioleoyl-phosphatidate alone. However, salts were interfering. Increasing salt concentrations stimulated the enzyme activity in the absence and inhibited the activity in the presence of the detergent. This was particularly obvious with potassium and sodium phosphate. Whether the salts act by interfering with the organization of the mixed micelles or with the binding of the enzyme to the substrate/detergent micelle is unclear at present.

On the basis of the results obtained in this investigation, it could thus be concluded that it is important to take differences in ionic strength, Triton X-100 concentration, and phosphatidate species into account when comparing PAP activities measured by different assay protocols, to investigate the influence of these factors when setting up an assay for PAP, and to be aware that salts and detergents could be carried over into the assay with enzyme preparation or substrate in amounts large enough to affect the results. γ-^{33}P-ATP could be used instead of γ-^{32}P-ATP in order to prepare a substrate for PAP with longer radioactive half-life and less potential health hazard.

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Comparison of the Structural and Functional Effects of Monomeric and Dimeric Human Apolipoprotein A-II in High Density Lipoprotein Particles

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ABSTRACT: High density lipoprotein (HDL) is thought to play a significant role in the process of reverse cholesterol transport. It has become clear that the apolipoprotein (apo) composition of HDL is important in determining the metabolic fate of this particle. The major proteins of human HDL are apoAI and APOAII; the latter protein is a disulfide-linked dimer in humans and higher primates but monomeric in the other species. The consequences of the apo Cys6-Cys6 disulfide bridge in apoAII for human HDL structure and function are not known. To address this issue, the influence of the Cys6-Cys6 disulfide bridge on the interaction of human apoAII with palmitoyl–oleoyl phosphatidylcholine has been studied. The size and valence of a series of homogeneous discoidal complexes containing either monomeric (reduced and carboxymethylated) or dimeric apoAII have been determined, and their ability to remove cholesterol from rat Fu5AH hepatoma cells grown in culture has been compared. The apoAII dimer and monomer form discoidal complexes of similar size, with twice as many of the latter molecule required per disc. Removal of the disulfide bond influences the stability of the helical segments around the edge of the disc as seen by a decrease in α-helix content of the monomeric protein. The discoidal particles containing the monomeric form of apoAII are somewhat more effective than particles containing either dimeric apoAII or apoAI in removing cellular cholesterol. Overall, reduction of the disulfide bridge of apoAII probably does not have a major effect in the determination of HDL particle size *in vivo*. It follows that the evolution of the Cys6-Cys6 disulfide bond in higher primates probably has not had a major effect on the function of the apoAII molecule.
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High density lipoprotein (HDL) particles function *in vivo* as a carrier of cholesterol in the reverse cholesterol transport pathway by which cholesterol is transported from the periphery to the liver for excretion (for reviews see Refs. 1 and 2). Consequently, increased plasma concentrations of HDL tend to prevent atherogenesis and are inversely correlated with the incidence of premature coronary artery disease (3). The major proteins of human HDL are apolipoproteins (apo) AI and AII. There are subspecies of HDL comprising particles that variously contain either apoAI (LpAI) or apoAII (LpAII) alone, or a mixture of both proteins (LpAI + AII) (1–4). It seems that apoAII interferes with the function of apoAI in reverse cholesterol transport because expression of high levels of apoAII (either mouse or human) does not prevent formation of atherosclerotic lesions in transgenic mice (5,6). Studies with mice transgenic for human apoAII indicate that this protein participates in determining HDL particle size (7); this effect may be the reason for the apparent modulation by apoAII of reverse cholesterol transport in these animals. Results from cell culture experiments using either natural or reconstituted HDL particles show that LpAI, LpAII, and LpAI + AII can sustain cellular cholesterol efflux (8,9); depending upon the cell type, LpAII can be somewhat less effective than LpAI (9). In summary, the current view of the function of apoAII is that it seems not to be essential in HDL metabolism but that it affects HDL functional states and probably contributes to atherogenesis (10).

In humans, amino acid 6 of apoAII is cysteine whereas this is not the case in other mammals including monkeys (10). Consequently, plasma apoAII is a disulfide-linked dimer of two 77-amino acid chains in humans but monomeric in the other species. This raises the question of the role of the apoAII Cys6-Cys6 disulfide bridge in human HDL structure and function. Dimeric apoAII (apoAII-D) binds well to phospholipid and, in fact, with higher affinity than apoAI perhaps because apoAII is more hydrophobic (11). Consistent with the enhanced binding affinity, dimeric apoAII can displace apoAI from HDL particles (12–15). It has been shown that reduction and carboxymethylation of the Cys residues in apoAII to form monomeric apoAII (apoAII-M) does not prevent binding to phospholipid and formation of complexes (16). However, lipid-free monomeric apoAII binds less well than dimeric apoAII to plasma membranes from either rat liver

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Abbreviations: apo, apolipoprotein; apoAII-D, apolipoprotein AII-dimer; apoAII-M, apolipoprotein AII-monomer; BSA, bovine serum albumin; Cys, cysteine; DMPC, dimyristoyl phosphatidylcholine; FC, free cholesterol; GdnHCl, guanidine HCl; HDL, high density lipoprotein; PAGGE, polyacrylamide gradient gel electrophoresis; POPC, 1-palmitoyl, 2-oleoyl phosphatidylcholine; rHDL, reconstituted HDL; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.

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cells or bovine aortic endothelial cells (17). Consistent with this, monomeric apoAII can exchange more rapidly than the dimeric molecule between phospholipid vesicles (18). A comparison of the effects of adding HDL containing monomeric and dimeric apoAII to plasma preparations showed that the dimers inhibit formation of small HDL particles (19). However, despite these isolated observations, there has been no systematic comparison of the lipid interactions of monomeric and dimeric apoAII. Given recent developments in understanding how apoAI forms discrete subpopulations of particles when reconstituted with phospholipid (20–22), it is of interest to know how the monomer and dimer forms of apoAII participate in this process.

In this study, the influence of the Cys6-Cys6 disulfide bridge on the interaction of apo AII with palmitoyl–oleoyl phosphatidylcholine (POPC) is investigated. In addition, the abilities of the reconstituted monomer and dimer LpAII particles to function as acceptors of cellular cholesterol are compared. The results show that elimination of the disulfide bridge to form monomeric apoAII molecules does not affect the size distribution of the reconstituted HDL particles but has some effect on their ability to remove cellular cholesterol. The α-helical segments of apoAII are less stable when the Cys6-Cys6 disulfide bond is broken.

EXPERIMENTAL PROCEDURES

Materials. Sodium cholate was purchased from Sigma (St. Louis, MO). Egg yolk phosphatidylcholine, 1-palmitoyl 2-oleoyl phosphatidylcholine (POPC), and dimyristoyl phosphatidylcholine (DMPC) were purchased from Avanti Polar Lipids (Birmingham, AL). Guanidine HCl was obtained from Bethesda Research Laboratories (Bethesda, MD). Essentially fatty acid-free bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO). Dimethyl suberimidate was purchased from Pierce (Rockford, IL). All other reagents were analytical grade.

Methods. Isolation and purification of apolipoprotein AII. Human HDL was isolated from fresh plasma from normolipidemic subjects by a modification of the polyanion precipitation method of Burstein *et al.* (23). For every 1 vol of plasma, 0.1 vol of 4% (wt/vol) sodium phosphotungstate in water were added to precipitate low density lipoprotein and very low density lipoprotein. The precipitate was pelleted by centrifugation at 4°C for 10 min at 1800 rpm. The density of the supernate was adjusted to 1.21 g/mL with dry potassium bromide, and the solution was ultracentrifuged at 50,000 rpm in a Beckman 50.2 Ti rotor for 24 h. The total HDL fraction was dialyzed extensively against 4 L of saline in water [0.9% (wt/vol) NaCl and EDTA (2 mM)] and then against dilute saline in water [0.09% (wt/vol) NaCl and EDTA (2 mM)] at 4°C. After concentration to 20–40 mg protein per mL in a filtration cell (Amicon, Beverly, MA) with a molecular weight cut-off of 100 kDa, the HDL was delipidated with ethanol–diethyl ether as described by Scanu and Edelstein (24). ApoAII was isolated from the apo HDL by anion exchange chromatography on Q-Sepharose (25) and stored in lyophilized form at −70°C. Prior to use, purified apoAII was resolubilized in 6 M guanidine HCl and dialyzed extensively against a 10 mM Tris buffer that contained 150 mM NaCl (pH 8.0).

Reduction and carboxymethylation of ApoAII. In human plasma, mature apoAII (17 kDa) exists as a homodimer with each subunit being about 8 kDa connected by a disulfide linkage. Monomeric apoAII was prepared by a modification of the method of Jackson *et al.* (26). Briefly, lyophilized, lipid-free apoAII (20 mg) was dissolved in Tris buffer containing 3 M guanidine HCl and 0.02 M dithioerythritol at pH 8.6. This mixture was incubated at 30°C for 6 h under nitrogen. The reduced form of the protein was then carboxymethylated by the addition of iodoacetic acid (50 mg) and incubation under the same conditions for 1 h. The mixture was then desalted on a Bio-Gel P-2 column (Bio-Rad Laboratories, Hercules, CA) (1.5 × 30 cm) in 0.1 M ammonium bicarbonate buffer. Those fractions found to contain protein by absorbance at 280 nm were pooled and lyophilized. Analysis by SDS–polyacrylamide gel electrophoresis (PAGE) showed that the reduced and carboxymethylated apoAII contained <1% unreacted apoAII dimer. As with the dimeric form of apoAII, the monomeric form was resolubilized in 6 M guanidine HCl and dialyzed extensively against Tris buffer before use.

Preparation and characterization of reconstituted HDL (rHDL) particles. All POPC-containing discoidal particles were reconstituted using the cholate removal method originally described by Bonomo and Swaney (27) and modified by Sparks *et al.* (28). The desired amount of POPC in chloroform (see Table 1) was added to a 15 mL borosilicate tube and dried under nitrogen. Tris buffer was added to give a POPC concentration of 20 mM and vortexed. Enough sodium cholate was added to achieve a molar ratio of POPC/cholate of 0.74 followed by vortexing. The resulting solution was incubated at 37°C and vortexed every 15 min for 1.5 h. The desired amount of either apoAI [isolated as described previously (25)] or apoAII (see Table 1) was added, and the mixture was diluted to 1 mg protein per mL and then incubated for 1 h at 37°C. The sodium cholate was removed by incubation with hydrated Biobeads (1 g of Biobeads/2 mg sodium cholate) for 3 h at 4°C. The Biobeads were then removed by filtration through a 0.22 μm filter. The resulting complexes were concentrated to about 1–2 mg protein per mL in Centricon 30 concentrators (Amicon) and stored at 4°C for no more than 2 wk.

DMPC-containing discoidal particles were prepared by mixing lipid and protein directly at the desired ratio. DMPC was dried from a chloroform solution under nitrogen to a thin film on the walls of a test tube. Traces of remaining solvent were removed by drying for 1 h under vacuum. The proteins in Tris buffer were added to the lipid film and bath-sonicated for 5 min at the gel to liquid crystal transition temperature of the lipid (24°C). The DMPC and the apo formed characteristic discoidal complexes. In a typical experiment, DMPC and protein were dispersed to give 2.5:1 (w/w) DMPC–protein discoidal complexes.

The size and homogeneity of the particles were estimated

TABLE 1
Comparison of POPC/apoAI, POPC/apoAII-Dimer, and POPC/apoAII-Monomer[a] Discoidal Complexes

	Molar ratio			Number apo mol per particle[b]			Diameter (nm)[c]		
Complex	POPC AI	POPC AII-D	POPC AII-M	AI	AII-D	AII-M	AI	AII-D	AII-M
D1	37:1	20:1	10:1	2	4	8	7.3 ± 0.2	8.0 ± 0.2	8.3 ± 0.3
D2	75:1	38:1	18:1	2	4	8	9.3 ± 0.3	9.3 ± 0.2	9.2 ± 0.3
D3	107:1	58:1	29:1	2	4	8	10.0 ± 0.3	9.8 ± 0.3	9.4 ± 0.3
D4	145:1	96:1	48:1	2–3	4	8	12.8 ± 0.3	10.5 ± 0.3	10.3 ± 0.3

[a]Reduced and carboxymethylated monomeric apoAII (apoAII-M).
[b]Determined from SDS–polyacrylamide gel electrophoresis of dimethylsuberimidate crosslinked complexes.
[c]Hydrodynamic diameter determined from nondenaturing gradient gel electrophoresis using reference globular proteins. Analysis by unpaired *t*-test indicated that the particle diameters for each type of complex were not significantly different except that the apoAI D1 particle was significantly ($P < 0.05$) smaller than the other D1 particles and the apoAI D4 particle was significantly larger ($P < 0.001$) than the other D4 particles. POPC, 1-palmitoyl, 2-oleoyl phosphatidylcholine.

by electron microscopy and nondenaturing PAGE. Negative stain electron microscopy was performed at 80 K magnification as described by Forte and Nordhausen (29). Mean particle dimensions of 100 particles were determined from each negative. The hydrodynamic diameters of discoidal rHDL particles were estimated by Phast electrophoresis using precast 8–25% polyacrylamide gels (Pharmacia, Biotech Inc., Piscataway, NJ) under nondenaturing conditions (28). The particle migrations into the gel were determined to within 0.1 mm by digitizing the gel with a Mustek 105 hand scanner (Mustek, Taiwan) followed by computer image analysis (Sigma Scan/Image; Jandel Scientific, San Rafael, CA). Intact rHDL particles were chemically analyzed where appropriate using the Markwell modification of the Lowry protein assay (30) while phospholipids were determined as inorganic phosphorus by the method of Sokoloff and Rothblat (31). The number of protein molecules per particle was determined by crosslinking the apoAII with dimethyl suberimidate as reported by Swaney (32). The electrophoretic mobilities of HDL particles were measured on Beckman (Fullerton, CA) Paragon preformed 0.5% agarose gels, and the net number of negative charges per complex (valence) was calculated as described by Sparks and Phillips (33). The average α-helix content of either lipid-bound or lipid-free apoAII was determined by circular dichroism spectroscopy using a Jasco J41A spectropolarimeter (Jasco Inc., Easton, MD). Spectra were measured at 25°C in a 0.1-cm path length quartz cuvette as described previously (22). The percentage α-helix was determined from the molar ellipticities at 222 nm (34). The effect of guanidine HCl on the α-helix content of the rHDL particles was used to obtain the free energy of unfolding of apoAII as described previously (22).

Interaction of proteins with phospholipids: (i) monolayers. The relative affinities of the dimeric and monomeric apo AII for the lipid–water interface were investigated using a surface balance technique. Employing published procedures (21,22,35), an insoluble monolayer of egg yolk PC was spread at the air–water interface in a circular Teflon dish containing 80 mL of phosphate-buffered saline (pH 7.0) at room temperature. The surface pressure (π) was monitored by the Wilhelmy plate technique using a mica plate connected to a Cahn RTL recording electrobalance (Cahn Instruments, Inc., Cerritos, CA). Sufficient egg yolk PC was spread from 9:1 (vol/vol) hexane/ethanol solution to give an initial surface pressure (π_i) in the range 5–30 dynes/cm. Proteins dissolved in the above buffer containing 1.5 M GdnHCl were injected into the subphase to give an initial concentration of 5×10^{-5} g/dL; a small Teflon tube which projected through the monolayer into aqueous subphase was used for this injection so that the egg yolk PC monolayer was not disrupted. The presence of GdnHCl ensured that the protein molecules were initially present as random coil monomers. The protein molecules renatured in the subphase as the GdnHCl was diluted to a final concentration of ≤1mM. The solution was stirred continuously with a magnetic stirrer, and the increase in surface pressure ($\Delta\pi$) with time was recorded until a steady-state value of $\Delta\pi$ was obtained. The steady-state $\Delta\pi$ values were plotted as a function of π_i. A linear extrapolation to $\Delta\pi = 0$ dyn/cm gave the exclusion pressure, or the value of π_i (±1 dyn/cm) at which the proteins were no longer able to penetrate into the egg yolk PC monolayer.

(ii) Multilamellar liposomes. DMPC in chloroform was dried in borosilicate tubes under nitrogen and then under vacuum for 1 h. Tris buffer (0.01M Tris, 1.0 mM EDTA and 0.15 M NaCl, pH 8.2) was added to give a lipid concentration of 10 mg/mL and vortexed to form a turbid suspension of multilamellar liposomes. The abilities of the proteins to solubilize the DMPC liposomes into small discoidal particles were examined by monitoring the optical density at 400 nm. An aliquot of a 10 mg/mL solution in 1 M guanidine-HCl of the apoAI or apoAII preparations was added to the DMPC liposomes to attain a weight ratio of 2.5:1 DMPC/protein. The guanidine-HCl was present to prevent self-association and to ensure that all of the apoproteins were in a common physical state. Tris buffer was added quickly to give a final volume of

1.4 mL and a final guanidine-HCl concentration of 70 mM. After mixing, the absorbance of the mixture at 400 nm was recorded as a function of time on a Varian DMS90 spectrophotometer (Palo Alto, CA). All solutions were maintained at 24° ± 0.2°C. The absorbance of DMPC liposomes (2.5 mg/mL), containing 70 mM guanidine-HCl in a final volume of 1.4 mL, was recorded as a control.

Efflux of cellular cholesterol. Rat Fu5AH hepatoma cells were used to monitor the unesterified (free) cholesterol (FC) efflux efficiencies of discoidal complexes of DMPC and various apoproteins. The methods for the efflux assay as well as the post experiment work-up were as previously reported (35) with the following exceptions. The Fu5AH cells were plated 6 d prior to the experiment in 12-well cell plates (22 mm) at 75–100 × 10^3 cells per well. 2 μCi/well [^{3}H]-FC in bicarbonate-buffered MEM with 3% fetal calf serum was used for the cell labeling procedure. After a brief wash, the efflux measurements were initiated by the application of 1.0 mL/well of the test medium containing 0.5% BSA and the acceptor particle present at 100 μg protein/mL. Typical cell protein values were between 250 and 300 μg protein per well. The fractional release of cellular FC determined experimentally was analyzed as described in detail for these systems previously (35). The fractional efflux at any time was corrected for the efflux observed to control medium that did not contain any added acceptor particles. The kinetic analysis to derive the apparent rate constant (k_e) for the efflux process assumed a closed system in which FC exists in one of two kinetic pools, either the cellular FC pool or the acceptor FC pool. The halftimes ($t_{1/2}$) for efflux was derived from the relationship ($t_{1/2} = \ln 2/k_e$).

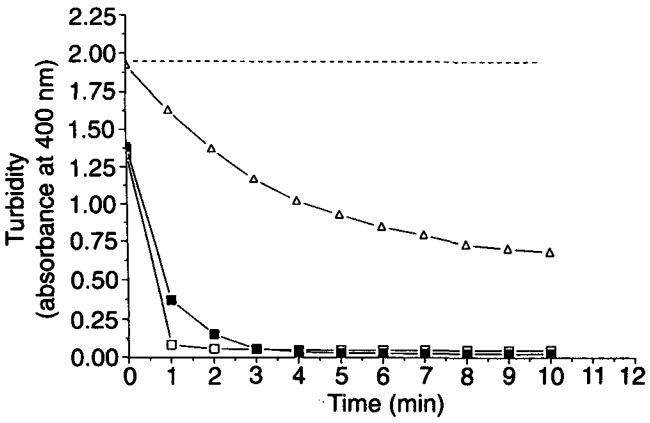


FIG. 1. Comparison of the abilities of apoAI, AII-D, and AII-M to solubilize DMPC liposomes. The absorbance at 400 nm of control dimyristoyl phosphatidylcholine (DMPC) liposomes [(----), 2.5 mg in 1.4 mL buffer] at 24°C was measured every minute up to 10 min; 1 mg apoAI (△), 1 mg apoAII-D (■), or 1 mg apoAII-M (□) were added to 2.5 mg DMPC liposomes, final volume 1.4 mL, and the absorbance was measured for up to 10 min.

RESULTS

Characterization of the lipid binding affinity of apoproteins. The lipid binding affinities of apoAI, apoAII-D, and apoII-M were compared by studying the changes in turbidity of suspension of DMPC multilamellar liposomes as a function of time (Fig. 1). The rates of interaction of the apoproteins with the DMPC liposomes were in the order: apoAI < apoAII-D, apoAII-M. The exclusion pressures for penetration into an egg PC monolayer for apoAI, apoAII-D and apoAII-M were 34, 36, and 34 dyn/cm, respectively. These values confirm that because of its higher hydrophobicity apoAII-D is slightly more surface-active than apoAI (cf. 11). However, disruption of the disulfide bond in apoAII to form apoAII-M slightly reduces the lipid affinity of apoAII-D.

Characterization of the size and composition of rHDL particles. A series of well defined complexes of POPC and apoAI, apoAII-D, or apoAII-M were prepared (Table 1). These particles were designed to contain different numbers of protein molecules. Electron microscopy showed these rHDL particles to be highly homogeneous and discoidal in appearance. Table 1 shows clear increases in particle hydrodynamic diameter in the range 7–13 nm for apoAI-containing particles and 8–10 nm for the apoAII-containing particles, as the rHDL phospholipid to protein ratio was increased. Chemical cross-linking of the protein molecules showed that all the discoidal complexes contained two molecules of apoAI/particle except for the 12.8 nm particle which probably contained three molecules (36). In contrast, in the apoAII-D and apoAII-M-containing complexes, the D1 and D2 particles contained four and eight protein molecules, respectively. The D3 and D4 complexes had a protein content of four or eight molecules apoAII-D and apoAII-M per particle, respectively. The particles appeared homogeneous on native polyacrylamide gradient gel electrophoresis (PAGGE), each exhibiting a single, discrete band (data not shown). Agarose gel electrophoresis was used to determine the effect of reducing the disulfide bond of the dimeric apoAII on the charge of the particles. The particle valences varied between −5.6 and −8.6e for the apoAII-D and apoAII-M particles (Table 2). Previous studies have shown that variations in apoAI-containing rHDL particle valences of up to 2e can be caused by a change in particle size [cf. Table 1 and (22)]. It is apparent from Table 2 that there is a similar effect of particle size on the charge of the particles for the discs containing either apoAII-M or apoAII-D. The fact that the valences of apo AII-M and apoAII-D in equivalent discoidal complexes are generally similar (Table 2) indicates that the removal of the disulfide bridge does not alter the net charge of the protein molecule. Furthermore, the differences in secondary structure of apoAII-D and apoAII-M in the discs (see below) do not affect the overall ionization of the charged amino acid sidechains.

ApoAII secondary structure and conformational stability. CD was used to determine the average α-helix content of both free and lipid-bound apoAII-D and apoAII-M. In this study, the α-helix contents of lipid-free apoAII-D and apoAII-M were considerably lower, 37 and 18%, respectively, than the value reported for lipid-free apoAI (46%, 22). When compared to apoAI complexed with POPC, the apoAII/POPC

TABLE 2
Physical Characteristic of Discoidal POPC/apoAI, POPC/apoAII-Dimer, and POPC/apoAII-Monomer Particles with Increasing Phospholipid to Protein Molar Ratio

	% α-helix[a]			Valence (e)[b]		
Complex	AI	AII-D	AII-M	AI	AII-D	AII-M
D1	62	51	39	−5.8 ± 0.2	−5.6 ± 0.2	−5.9 ± 0.2
D2	67	58	47	−7.7 ± 0.4	−6.3 ± 0.3	−6.3 ± 0.3
D3	82	61	51	−7.7 ± 0.4	−6.6 ± 0.3	−6.2 ± 0.3
D4	84	63	61	−10.9 ± 0.0	−8.6 ± 0.2	−7.5 ± 0.3

[a]Values calculated from the molar ellipticity at 222 nm ± 5% (SD).
[b]The number of excess negative charges per particle in electronic units derived from migration in a 0.5% agarose gel at pH 8.6. Analysis by unpaired *t*-test indicated that the valences of apoAII-D and apoAII-M were not significantly different in the D1–D3 complexes but were different ($P < 0.01$) in the D4 particles. See Table 1 for abbreviations.

discs had generally lower α-helix contents (Table 2). However, in all cases there was an increase in α-helix content directly related to increases in POPC content and particle diameter (Table 2). It is apparent that the smaller apoAII-M-containing particles had lower α-helix contents than the equivalent apoAII-D particles. The stabilities of the α-helices in apoAII-M and apoAII-D were monitored in the presence of increasing concentrations of GdnHCl. The particles containing apoAII-D were progressively denatured by GdnHCl, and the process was complete at approximately 5 M whereas equivalent discoidal complexes made with apoAII-M required about 3 M GdnHCl to fully denature (data not shown). The free energies of denaturation of apoAII-D increased from 2.4 ± 0.22 to 3.5 ± 0.21 kcal/mol as the discoidal complexes increased in size from D1 to D4. Such an increase in helix stability with increasing POPC/apoAI disc size has been reported previously (from 1.4 ± 0.2 to 3.0 ± 0.2 kcal/mol) (22). The equivalent values for the apoAII-M discs were 0.7 ± 0.2 to 1.4 ± 0.1 kcal/mol; this indicates that disruption of the disulfide bond in apoAII-D tends to destabilize the α-helical segments of the molecule, presumably due to reduced interaction between amphipathic α-helices.

Efflux of cellular cholesterol to ApoA-containing rHDL particles. The abilities of the discoidal D3 DMPC/apoA particles (Table 1, described in Methods) to act as acceptors of cell plasma membrane cholesterol at 100 μg phospholipid per mL were compared using rat Fu5AH hepatoma cells that had been labeled with [^{3}H]FC. Figure 2 demonstrates that the DMPC/apoAII-M particles display an enhanced ability to accept cellular FC relative to the DMPC/apoAI or DMPC/apoAII-D particles. The $t_{1/2}$ for FC efflux from Fu5AH to the rHDL made with apoAI, apoAII-D, and apoAII-M were 9.6 ± 0.5, 10.3 ± 1.5, and 3.8 ± 0.3 h, respectively. Analysis by unpaired *t*-test indicates that the $t_{1/2}$ for apoAI and apoAII-D are not significantly different whereas the $t_{1/2}$ for apoAII-M is significantly lower ($P < 0.005$) than either of the prior values. The FC efflux efficiency of the apoAI-containing particles is consistent with that seen in previous studies (37).

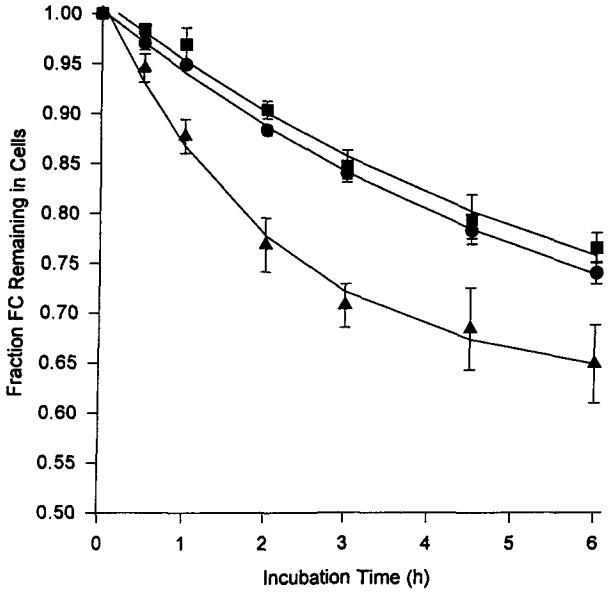


FIG. 2. Time course of [^{3}H]free cholesterol efflux from rat Fu5AH hepatoma cells to discoidal reconstituted high density lipoprotein (rHDL) containing various apoproteins. Rat Fu5AH hepatoma cells that had been trace-labeled with [^{3}H]free cholesterol in 22-mm tissue culture wells were incubated for 6 h at 37°C in a humidified incubator (5% CO_2) with 1.0 mL of test medium containing 0.5% bovine serum albumin and the rHDL containing the indicated apoprotein at 100 μg of protein/mL. The rHDL particles (D3 complexes, Table 1) were: DMPC/apoAI (●), DMPC/apoAII-dimer (■), and DMPC/apoAII-monomer (▲). The vertical axis indicates the fraction of initial labeled free cholesterol (FC) that remained in the cell at the designated times. Each point represents the mean of three cell wells. The error bars represent 1 SD. All curves were obtained by fitting the entire time course to the model for tracer equilibration between two pools. See Figure 1 for other abbreviation.

DISCUSSION

This study examined the effects of reducing the apoAII Cys6-Cys6 disulfide bridge on the structure of rHDL and also on the function of this particle in cholesterol efflux. These two effects are discussed in turn below.

(i) Structure. Spontaneous clearing of DMPC liposomes by direct addition of protein, in an aqueous solution, occurs more rapidly for apoAII than for apoAI (Fig. 1). This is in agreement with previously published data from this laboratory showing apoAII-D to have a higher surface activity than apoAI (11). The faster rate of reaction of the monomeric apo A-II compared to dimeric apo A-II (Fig. 1) is presumably due to its expected higher diffusion coefficient. Also, binding to lipid–water interfaces, as measured by monolayer exclusion pressure, is slightly less for the dimeric protein. Apo AI, apoAII-M, and apoAII-D can all form discoidal complexes of similar size with POPC; these discoidal particles are organized with the amphipathic, α-helical, segments of the apoprotein molecules covering the POPC acyl chains at the disc edge (20–22). Table 1 shows clear increases in the particle hydrodynamic diameter as the rHDL phospholipid to protein ratio increases for these complexes. For any particle of

given size, twice the number of apoAII-D molecules, compared to apoAI, are required to achieve particle stability. This suggests that the amphipathic α-helices present in two molecules of apo AII-D can cover as many PC acyl chains at the disc edge as the helices present in one apoAI molecule. This is consistent with the observation that two apo AII-D molecules can displace one apoAI molecule from either discoidal or spherical HDL particles (12,13). Since two times as many apoAII-M as apoAII-D molecules can form discs of the same size with POPC (Table 1), it is apparent that HDL particle size is not affected by the presence of the disulfide bridge. It follows that the role of apoAII in determining HDL particle size *in vivo* (7) is independent of the Cys6-Cys6 disulfide bond.

However, although equal masses of apoAII-M and apoAII-D can form rHDL particles of the same size, the information on secondary structure (α-helix content) and conformational stability (free energy of denaturation) indicates that removal of the disulfide bond influences the packing of the apoAII molecule around the edge of the POPC disc. The only major structural effect of reducing the apoAII disulfide bridge is on the stability of the helical segments. This effect presumably arises from the reduced opportunity for intramolecular helix-helix interactions in the monomeric molecule. It is likely that these helical segments, which are less resistant to denaturation by GdnHCl in apoAII-M than in apoAII-D, are more loosely packed around the edge of the disc (see below). The greater exchangeability of apoAII-M molecules compared to apoAII-D molecules (19) is consistent with this concept.

(ii) Function. The difference in helical packing between POPC discs containing apoAII-M and apoAII-D might be expected to influence the abilities of these particles to accept cellular cholesterol because a dependence of efflux on rHDL structure has been demonstrated (34). There is general agreement that the plasma membrane pool of cholesterol leaves the cell *via* the aqueous diffusion mechanism (37–40). Transfer by this mechanism occurs by a two-step process that involves the desorption of a cholesterol molecule from the membrane and subsequent diffusion through the aqueous phase until it incorporates into a phospholipid-containing acceptor particle. At low acceptor particle concentration (≤100 μg POPC/mL), the rate of cellular cholesterol transfer to an extracellular HDL particle depends upon the rate constants for (i) desorption from the plasma membrane and (ii) collision and incorporation of the desorbed cholesterol molecule into the HDL particle. The second term is affected by the diffusion coefficient of the acceptor particle and the fraction of HDL-cholesterol molecule collisions that result in incorporation of a cholesterol molecule into the HDL particle. For various acceptor particles of the same size but different structure, only the fraction of successful collisions can differ because their diffusion coefficients are the same. It follows that the greater rate of efflux of cellular cholesterol to the POPC/apoAII-M disc depicted in Figure 2 must occur because of such an effect. Presumably, the looser arrangement of the α-helices around the edge of this disc relative to either the apoAI/POPC or apoAII-D/POPC disc leads to a greater fraction of the collisions with cholesterol molecules diffusing in the extracellular aqueous phase resulting in successful incorporation of the cholesterol molecule into the disc. The observation that the POPC/apoAI and POPC/apoAII-D discs have similar capabilities of removing cellular cholesterol is consistent with prior comparisons of LpAI and LpAI + AII isolated from human plasma (8).

Conclusion. Overall, reduction of the disulfide bridges in apoAII seems not to have a major effect. Discoidal particles of similar size can be made with apoAII-D and apoAII-M with twice as many of the latter molecules required. The ability of apoAII to modulate HDL particle size is not affected by the presence or absence of the Cys6-Cys6 disulfide bond. This conclusion is consistent with studies of the effects on HDL of overexpression of apoA-II in transgenic mice. Thus, overexpression of either dimeric human apoA-II (7) or monomeric human apoA-II where Cys6 was mutated to Ser (41) has no significant effect on either the levels or particle size of the major HDL species. In contrast, overexpression of mouse apoA-II leads to increases in both HDL levels and particle diameter (42). These results indicate that while removal of the Cys6-Cys6 disulfide bond in apoA-II has no effect on HDL metabolism in mice, variations elsewhere in the amino acid sequences of the human and murine proteins lead to significant variations in function.

With regard to the initial step in the reverse cholesterol transport process, the data in the paper suggest that a discoidal particle containing apoAII-M is somewhat more effective than either apoAI- and apoAII-D containing particles in removing cellular cholesterol. However, overall it seems that the evolution of the Cys6-Cys6 disulfide bridge in higher primates probably has not had a major effect on the function of the apoAII molecule.

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Identification of Fatty Acid Methyl Ester as Naturally Occurring Transcriptional Regulators of the Members of the Peroxisome Proliferator-Activated Receptor Family

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ABSTRACT: The nuclear hormone receptors NUC-1 (PPARδ) and PPARα are members of the peroxisome proliferator-activated receptor (PPAR) family. The members of this receptor family are activated by agents that stimulate peroxisome proliferation, free fatty acids, prostaglandin J2 metabolites, and agents considered for the therapy of insulin-independent diabetes mellitus. To identify putative physiological agents that activate NUC-1, we tested the ability of acetone extracts of various rat tissues to activate the transcription of an MMTV-luciferase reporter gene, *via* a GR/NUC-1 hybrid receptor. GR/NUC-1 contains the ligand binding region of the NUC-1 receptor and the DNA binding domain of the glucocorticoid receptor. Using this assay, we found stimulatory activity in the pancreas, which upon purification and characterization was identified as methyl-palmitate, known to be enriched in pancreatic lipids. In addition, we determined that ethyl esters of palmitic and oleic acids are also potent activators of this receptor. Thus, fatty acid ester formation may control the cellular concentrations of fatty acids, and acyl-ester formation may play a role in the control of metabolic pathways and the activation of the PPAR.
Lipids 31, 1115–1124 (1996).

The nuclear hormone receptor NUC-1 (PPARδ) is a member of the steroid hormone receptor superfamily and a member of the peroxisome proliferator-activated receptor (PPAR) family that we cloned from the human osteosarcoma SAOS/B10 cells (1,2). The PPAR family contains the three mammalian receptors PPARα, PPARγ and NUC-1 (PPARδ) (1–3), and three xenopus receptors xPPARα–γ (4). Based on amino acid sequence similarity, NUC-1 belongs to this family of receptors, but its amino acid sequence is not close enough to xPPARβ to be the mammalian homologue of this receptor. The members of the PPAR family are activated by molecules that induce peroxisome proliferation (1–4). Several of these synthetic molecules, which include WY-14643, nafenopin and clofibric acid, are used for the therapy of hyperlipidemia. It was also shown that free fatty acids, such as linoleic, arachidonic, and oleic acid can specifically activate the mPPARα and the NUC-1 receptors (2,4–9). Thus, both the fatty acids and drugs for the treatment of hyperlipidemia can induce peroxisome proliferation and lower the levels of triglycerides in the blood. mPPARα, which is abundant in the liver, has therefore been proposed to participate in the regulation of fatty acid metabolism. Moreover, it was demonstrated that the PPARγ and PPARα and their ligands play a key role in adipocyte differentiation (10,11). Recently, it was shown that prostaglandin J2 metabolites bind and preferentially activate the PPARγ subtype (12,13). Furthermore, it was shown that thiazolidinedione antidiabetic agents that improve glucose sensitivity in noninsulin-dependent diabetes mellitus activate the members of the PPAR family (12–14).

In order to find putative physiological agents that modulate transcription *via* the NUC-1 receptor, we tested the ability of acetone powders of various rat tissues to activate the transcription of the MMTV-luciferase reporter gene, driven by the GR/NUC-1 hybrid receptor that contains the ligand binding region of the hNUC-1 receptor and the DNA binding domain of the glucocorticoid receptor (GR). We found that acetone powders of rat intestine and pancreas contain a substance that specifically activates the transcription mediated by the GR/NUC-1 receptor. We identified this substance as the methyl ester of palmitic acid. In addition, we show that the NUC-1 receptor can mediate the transcription from a reporter gene that is under the control of the peroxisome proliferator-responsive regulatory element of the rat Acyl-CoA oxidase gene promoter (4).

MATERIALS AND METHODS

Chemicals. Acetone powders of various rat tissues and the various fatty acids were obtained from Sigma (St. Louis,

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Abbreviations: DMSO, dimethylsulfoxide; GR, glucocorticoid receptor; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR hormone response element; UV, ultraviolet; WY-14643, 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio)acetic acid.

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MO). WY-14643 (4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio) acetic acid was obtained from Medicinal Chemistry Laboratories (Merck Frosst, Quebec, Canada).

Plasmids. The reporter gene MMTV-luciferase was the plasmid pJA358, in which the firefly luciferase gene is regulated by an MMTV promoter that contains tandem repeats of the glucocorticoid hormone response element (2,8). The reporter plasmid MMTV\PPRE-luciferase is similar to the above MMTV-luciferase reporter gene in which glucocorticoid hormone response element was replaced with the peroxisome proliferator responsive region found in the acyl-CoA oxidase gene (PPRE) and is identical to the PPRE in the 2A-GST-CAT reporter gene (8). Plasmid pJ3NUC-1 is an expression vector containing the cDNA of the NUC-1 receptor (2,8). Expression plasmid pSV2wrec contains the native mouse GR (2). The expression vector pJ3GR/NUC-1 was the chimeric receptor in which the DNA binding domain of GR was fused with the ligand binding domain of NUC-1 receptor (2,8). Expression vector pJ3GR/PPARα contains the chimeric receptor in which the DNA binding domain of GR was fused to the ligand binding domain of mPPARα (8). Expression vector pJ3NER contains the cDNA for the native NER steroid hormone receptor (15). Expression vector pJ3GR/NER contains the hybrid receptor in which the DNA binding domain of the GR was fused to the ligand binding domain of NER receptor.

Ligand-dependent transcription assays. Ligand-dependent screening assays were as previously described (2). The reporter gene MMTV-luciferase was co-expressed with plasmids containing cDNA for the steroid hormone receptors in COS-7 cells. Ligands were added 30 min after addition of the calcium phosphate–DNA co-precipitate. After 18 h, the cells were washed and fresh ligands were added. Twenty-four hours later, cell extracts were prepared and assayed for luciferase activity. In some experiments, transfection was performed in 150-mm tissue culture dishes. After 18 h, cells were washed and seeded into 24 well plates. After attachment of the cells, the ligands were added and luciferase activity was tested two days later. All compounds were dried and dissolved in ethanol or DMSO and added to the assays at 500–1000-fold dilutions of concentrated stock solutions. The data is presented as the mean of triplicate or tetraplicate samples with the standard deviation shown for each data point. Each assay was repeated at least three times with similar results obtained for each experiment.

Purification of methyl ester of palmitic acid. Acetone powders, acetone insoluble material, of various rat tissues were obtained from Sigma. The powder (50 g) was dissolved in 1000 mL of a chloroform/water/ethanol mixture (50:50:1). After vigorous mixing for 10 min, the phases were separated by centrifugation at 5000 × *g*. The chloroform phase which contained 90% of the activity was dried under vacuum. The residue was dissolved in hexane and loaded onto a silica gel column followed by elution with hexane, chloroform, ethyl acetate, propanol, and ethanol. The hexane phase contained most of the agonistic activity and was further purified on semi-preparative C_{18} high-performance liquid chromatography (HPLC) and C_4 HPLC columns employing linear gradients of 40% acetonitrile/60% water to 90% acetonitrile/10% water and monitoring the ultraviolet 200 nm.

The activity of the various fractions was tested after evaporation of the solvents. The materials were dissolved in ethanol and tested in the ligand screening assays for activity. The final concentration of ethanol did not exceed 0.2% of the cell culture medium.

Identification of methyl palmitate. The structure of the purified compound that activated the GR/NUC-1 chimeric receptor was analyzed by nuclear magnetic resonance (NMR). Proton and carbon NMR spectra were recorded at room temperature on a Varian VXR-500S NMR spectrometer (Palo Alto, CA) operating at a proton frequency of 500 MHz and a carbon frequency of 125 MHz. The material present in fractions 41–47 (pool 2, Fig. 4) of the C_4 (HPLC) column was dissolved in 100 atom percentage D CD_3CN (Merck Isotopes, Darmstadt, Germany). Chemical shifts are referenced to internal tetramethylsilane.

RESULTS

Activation of NUC-1 receptor by acetone powders of rat organs. To identify the putative natural ligand for the NUC-1 receptor, we prepared a ligand-dependent transcription assay that has been successfully used for the identification of ligands for members of the steroid hormone receptor superfamily (1,2,16–18). The ligand binding domain of the NUC-1 receptor was fused to the DNA binding domain of the GR as previously described (1,2,8). The recombinant GR/NUC-1 receptor was then co-transfected with the MMTV-Luciferase reporter gene into COS-7 cells, and the effect of potential ligands on expression levels of luciferase enzyme was monitored as described in the Materials and Methods section. Since most of the ligands for members of the steroid hormone receptor superfamily are hydrophobic, we tested the ability of acetone powders from various rat tissues to activate the GR/NUC-1 chimeric receptor. We found that acetone powders of intestine and pancreas produced the largest stimulation of luciferase activity (Fig. 1A). Acetone powders of muscle, intestinal mucosa, and kidney had lower activity while no significant stimulation of the luciferase activity was observed in acetone powders of liver, heart, lung, placenta, spleen, and adrenal cortex. In order to test the activation specificity of GR/NUC-1 by these acetone powders, we tested their effects on the ligand-dependent transcription assays mediated by the GR/NER chimeric receptor. This receptor is the fusion product of the amino terminal region, including the DNA binding domain, of the GR and the ligand binding domain of NER, a nuclear receptor that is not related to the PPARα family, and for which no ligand has been identified (15). In these experiments, none of the acetone powders exhibited stimulation of the MMTV-Luciferase reporter gene (Fig. 1B), suggesting that the pancreas and intestine acetone powders activate specifically the GR/NUC-1 chimeric receptor.

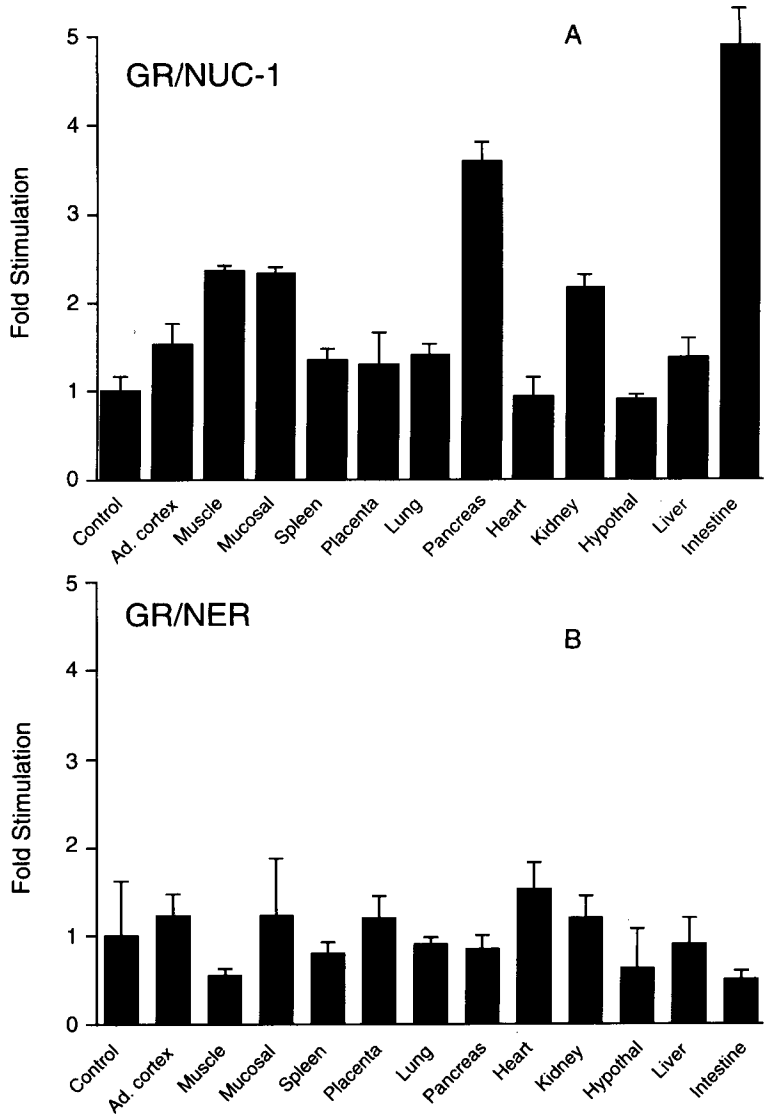


FIG. 1. Activation of GR/NUC-1 and GR/NER chimeric receptors by acetone powders of rat tissues. Acetone powders of various rat tissues were dissolved in dimethylsulfoxide (20 mg/mL) and cleared by centrifugation. Samples were added to the ligand-dependent transcription assays mediated by the chimeric receptors in cells transfected with GR/NUC-1 (A) or GR/NER (B). The abbreviations are: Ad. cortex, adrenal cortex; Mucosal, intestinal mucosa; Hypothal, hypothalamus.

Methyl palmitate is the active agent that stimulates NUC-1-dependent transcripion. We next examined the chloroform/water partition of the active component found in the acetone powders. The active material was added to a mixture of water and chloroform (1:1). After mixing, the two phases were separated by centrifugation and each phase was evaporated under vacuum. The subsequent residues were then dissolved in DMSO and tested for activity. We found that the chloroform phase contained 90% of the stimulatory activity present in the original acetone powder (Fig. 2). About 10% of the activity was present in the water phase. The chloroform-soluble material retained its specificity for activation of the GR/NUC-1 receptor. Neither the acetone powder nor the water-soluble material stimulated transcription mediated by the chimeric receptor GR/NER or by the wild-type GR (Fig. 2). The stimulation of GR/NUC-1 by acetone powders of intestine and pancreas was similar in magnitude and receptor specificity to that of 100 μM WY-14643. Furthermore, addition of the chloroform or acetone extracts to a transcription assay that contained only the MMTV-luciferase reporter gene produced no stimulatory activity (not shown).

To further purify the active ingredient, the chloroform-soluble material of the acetone powder was dissolved in hexane, loaded onto a silica gel column, and eluted with hexane, chloroform, ethanol, and methanol. The solvents were evaporated, samples of the residues were dissolved in DMSO and tested in the ligand-dependent transcription assay. Most of the activity was in the hexane fraction, although some activity was

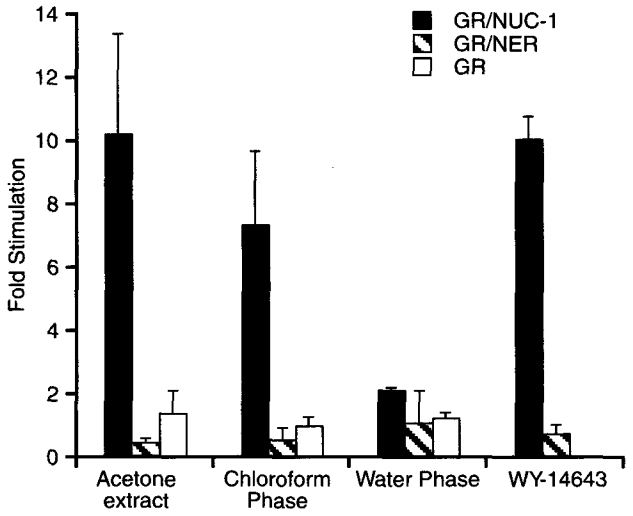


FIG. 2. Partition of GR/NUC-1-stimulation activity between acetone and water. One gram of pancreas acetone powder was added into a mixture of 50 mL water/chloroform (1:1). After mixing and vortexing, the two phases were separated by centrifugation and the separated water and chloroform phases were dried and dissolved in dimethylsulfoxide. Samples were added to the transfection assays as described in methods.

observed in other fractions. The hexane fraction was subjected to HPLC purification on a C_{18} column (Fig. 3A). Testing the C_{18} column fractions in the GR/NUC-1 ligand transcription assay, we found the highest activity was concentrated at fractions 61 to 68 which corresponded to the area of a major ultraviolet peak of fractions eluted at 44 to 48 min (Fig. 3). The active fractions, which on an analytical C_4 HLPC column exhibited similar ultraviolet absorption profile, were pooled and were further purified by HPLC on a C_4 column (Fig. 4A). The fractions 3–40, 41–47, and 48–69 were pooled, and the activity was found to be concentrated in pool 2 (fractions 41–47, Fig. 4B). The activity found in pool 2 was similar in magnitude to the activity found in the peak fraction (fraction 63) of the C_{18} column. HPLC analysis of the material in pool 2 fraction revealed that this material was relatively pure (Fig. 5A). Analysis of the proton NMR spectrum showed resonances characteristic of a saturated, straight-chain fatty acid (Fig. 5B). The 3-proton singlet at 3.6 ppm suggested that the fatty acid was esterified with a methyl group. The length of the fatty acid chain was obtained from integration of the proton resonances (NMR data are in the legend of Fig. 5B). A proton-decoupled carbon spectrum of the sample was found to be identical with that of an authentic sample of methyl palmitate (Fig. 5B). Thus the molecular structure of this compound was determined to be the methyl ester of palmitic acid. Since pool 2 fraction is of a pure material, the calculated concentration of methyl palmitate (from pool 2) in the transcription assay in Figure 4B was 52 μM and gave transactivation of GR/NUC-1 chimeric receptor (5.1-fold, Fig. 4) that was comparable to the stimulation magnitude of an authentic sample of 50 μM methyl palmitate (3.3-fold, Fig. 6).

To confirm that methyl palmitate is indeed capable of activating the GR/NUC-1 receptor, we tested commercially obtained methyl palmitate in the ligand-dependent transcription assay. Indeed, 99.9% pure methyl palmitate activated transcription mediated by the GR/NUC-1 receptor about threefold (Fig. 6A). Moreover, methyl ester of oleic acid was an equally potent agonist of the GR/NUC-1 receptor, whereas the free form of palmitic acid was less potent in the ligand-dependent transcription assay. In contrast, the free form of oleic acid was a potent activator. Furthermore, we found that an ethyl ester of oleic acid was also a potent activator in this assay (Fig. 6A). Similar results were observed with the chimeric receptor GR/PPARα which contains the ligand binding domain of the PPARα. The GR/PPARα was activated by methyl palmitate, ethyl palmitate, and the various forms of oleic acid (Fig. 6B). No such stimulation was observed when these molecules were added to ligand-dependent transcription assays mediated by other receptors with their cognate hormone response element such as GR/NER, GR, or retinoic acid receptors (not shown). We concluded, therefore, that the free fatty acids or their esters could specifically activate the proteins that belong to the PPAR subfamily.

Methyl palmitate activates the normal NUC-1 receptor. The PPAR family can recognize a specific DNA sequence within the promoter of the acyl-CoA oxidase gene that codes for a key enzyme in the metabolism of fatty acids. Therefore, we examined whether methyl palmitate can activate the transcription mediated by native NUC-1 receptor through the same DNA sequences (4,7,16). We replaced the glucocorticoid hormone response element found in the MMTV promoter with the PPAR hormone response element, thus forming a luciferase reporter gene that is under the control of the NUC-1 receptor. Co-expression of the wild-type NUC-1 receptor with this reporter gene resulted in a ligand-dependent stimulation of luciferase expression (Fig. 7). Either methyl palmitate or palmitic acid increased luciferase expression. In control experiments, addition of methyl palmitate or palmitic acid to cells cotransfected with the reporter gene containing the glucocorticoid hormone response element did not show ligand-dependent stimulation by NUC-1 receptor (Fig. 7). Moreover, methyl palmitate did not stimulate the luciferase expression mediated by the glucocorticoid receptor (not shown).

DISCUSSION

PPARα and NUC-1 belong to the steroid hormone receptor superfamily. They are activated by drugs for the treatment of hyperlipidemia and by fatty acids. Furthermore, these receptors stimulate transcription of reporter genes *via* response elements identified in promoters of genes that code for the enzymes that participate in peroxisomal fatty acid β-oxidation (4,8,9,19). These observations suggest that PPARα and NUC-1 play a role in the physiological regulation of fatty acid metabolism (1–4). We report here the identification of a putative natural activator of the members of the PPAR family. By testing acetone powders of various rat tissues in ligand-de-

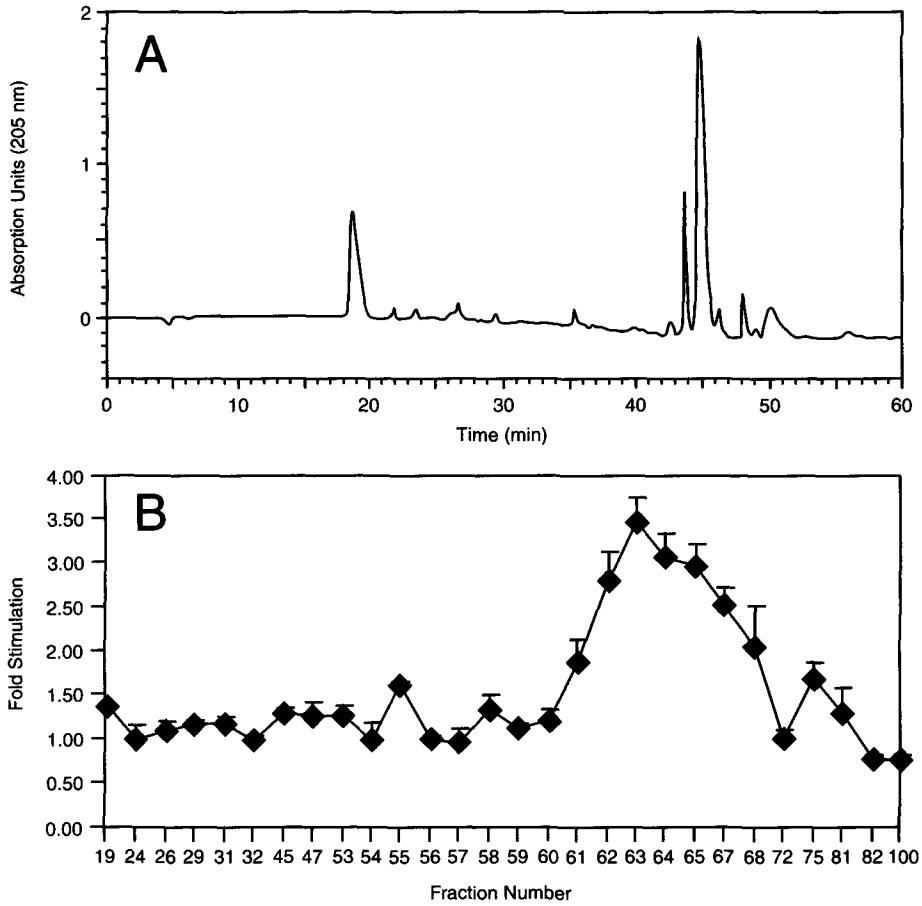


FIG. 3. Activation of GR/NUC-1 chimeric receptor by C_{18} high-performance liquid chromatography (HPLC) fractions of the pancreas powders. A 100-mg portion of the hexane fraction eluted from the silica gel column was dissolved in ethanol and was chromatographed on Vydac C_{18}, 10 μ, 300Å, 22.5 mm × 250 mm HPLC column employing a 40% acetonitrile/60% water to 90% acetonitrile/10% water linear gradient over 60 min at 5 mL/min flow rate (A). The various fractions were tested for the stimulation of transcription mediated by the GR/NUC-1 chimeric receptors (B). Column fractions were tested individually or as pool of fractions indicated in the figure as the number of the last fraction in the pool (1–19, 20–24, 25–26, 27–29, 30–31, 33–34, 35–45, 46–47, 48–53, 69–72, 73–75, 76–81, and 82–100) for further purification on C_4 HPLC column.

pendent transcription assays mediated by the GR/NUC-1 hybrid receptor, we discovered that intestine and pancreas powders contain substances that specifically stimulate the transcription *via* the NUC-1 ligand binding domain. The agonist specificity was reflected in the lack of stimulation of transcription mediated by the native GR or hybrid receptor GR/NER which have ligand binding domains different than that of PPAR. Moreover, the native NUC-1 that does not recognize the MMTV reporter gene did not transactivate transcription by the extracts. After purification, we identified the major active material in acetone powder of rat pancreas as the methyl ester of palmitic acid. This finding was confirmed by the ability of synthetic methyl palmitate to specifically activate both GR/NUC-1, the related GR/PPARα hybrid receptors, and the native NUC-1 receptor. Further experiments showed that the ethyl ester of palmitic acid was an equally potent activator of these receptors. Moreover, we found that the methyl- and ethyl esters of oleic acid were also potent activators of the PPARα and NUC-1 receptors. These observations are supported by the reports that synthetic methyl-ester compounds such as methyl clofenapate and LY-171883 were found to be potent activators of the members of the PPAR family (1,5). These compounds lack the carboxylic group that characterizes the various compounds that activate these receptors. These findings suggest that a free carboxylic group may not be required for the activation of these receptors, and that esters of the fatty acid may also act as activators of the PPAR family.

The natural occurrence of fatty acid methyl esters was reported only in few publications since, in most cases, fatty acid composition is analyzed after transmethylation of the organic extracts. However, there are confirmed reports in the literature of the isolation of fatty acid methyl esters from various human and animal cells and tissues (20–27). Moreover, the biochemical pathway for methyl-ester formation was determined to be the carboxyl methylation of fatty acids by

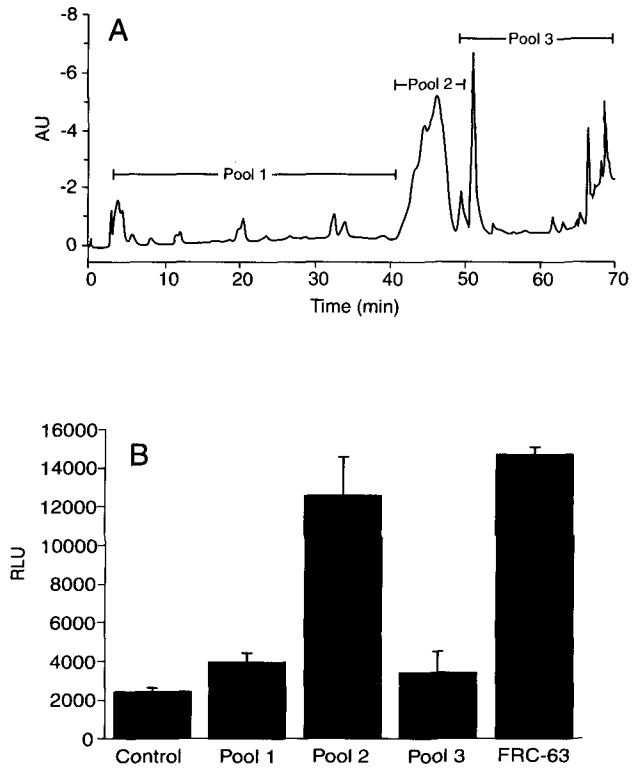


FIG. 4. Activation of GR/NUC-1 chimeric receptor by C_4 HPLC column fractions of the pancreas powders. Fractions 61–68 that corresponded to the C_{18} elution at 44 to 48 min were pooled. After evaporation, 16-mg portion was dissolved in 250 μM ethanol and was chromatographed on Vydac C_4 (Phenomenex, Torrance, CA), 5 μ, 300Å, 10 mm × 250 mm HPLC column employing a 40% acetonitrile/60% water to 90% acetonitrile/10% water linear gradient over 60 min at 5 mL/min flow rate, ultraviolet 200 nm (A). The various fractions were pooled as illustrated in part A, dried under vacuum, dissolved in ethanol (pool 1, 500 μg/150 μL; pool 2, 1010 μg/150 μL; pool 3, 390 μg/150 μL). Fraction 63 (FRC-63) represents a fraction from the peak activity eluted from the C_{18} HPLC column (45 min). See Figure 3 for company source and abbreviation.

S-adenosylmethionine (24–26). The formation of methyl esters of fatty acids was demonstrated in both cells and tissues, as well as membrane preparations incubated with a radioactive methyl group on methionine or *S*-adenosylmethionine. Furthermore, enzymatic activity that catalyzes the methyl hydrolysis was also described (27). Interestingly, the pancreas, from which we purified methyl palmitate, is one of the organs with the highest enzymatic activity for the production of fatty acid methyl esters (21,22,26).

The biochemical pathway for the formation of fatty acid ethyl esters has also been documented both *in vivo* and *in vitro* (28–32). Fatty acid ethyl esters are the end product of a nonoxidative pathway for ethanol metabolism in a variety of tissues from several species, including humans (28–31). The enzyme that catalyzes this reaction, fatty acid ethyl ester synthase, was identified in several tissues including lung, liver, and pancreas (28–32). It was shown that tissues that are most commonly damaged by alcohol abuse contain high levels of the fatty acid ethyl ester synthase (28). Moreover, the pancreas that was the source for our isolation of methyl palmitate is also rich in fatty acid ethyl ester synthase (30,33, 34). Interestingly, it was observed that palmitic acid increases the insulin release that was potentiated by a high glucose concentration from pancreatic β-cells (35). This effect appeared to be through an unknown glucose-dependent mechanism that is not mediated by the β-oxidation pathway.

An effort to isolate the natural activators of the members of the rat PPARα from human plasma led to the identification of palmitic acid, oleic acid, linoleic acid, and arachidonic acid (36). In our studies, the silica gel fractions obtained with solvents more polar than hexane, i.e., chloroform through methanol, contained compounds that activated the NUC-1 receptor. These fractions probably contain the free acid forms of the various fatty acids described above. Therefore, we elected to purify the compound(s) from the hexane fraction because of their higher specific activity.

It is of interest that methyl palmitate rather than the free acid form, which is the major fatty acid (over 50%) in mammals, was the PPAR family agonist purified from pancreas powders. The incorporation of the free acids into glycerol esters and fatty acid metabolism may reduce their effective concentration and reduces the pool of free fatty acids. Although there is no data in that matter, one may speculate that the formation of methyl ester of fatty acids will slow down the fatty acid metabolism and increase their cellular concentration and therefore increase the availability of fatty acids to the PPAR. Therefore, other cellular components such as uptake, cellular metabolism, or binding proteins may be the limiting factors in determining the response to the concentrations of exogenous methyl palmitate and palmitic acid. Further studies are needed to evaluate the *in vivo* effects of the esters of fatty acids. It was shown that administration of methyl palmitate suppresses the reticuloendothelial system and strongly inhibits the function of Kupffer macrophages in rats (37). It was also shown that methyl palmitate prevents liver damage mediated by the activation of Kupffer cells, after reperfusion that followed liver hypoxia (38). Treatment of rats with methyl palmitate also improved survival after orthotopic liver transplantation, probably by inhibiting the activity of Kupffer cells (39). It remains to be examined if the immunosuppressive effects of methyl palmitate are related to the activation of PPAR-type receptors. In this context, it is noteworthy that clofibrate, another PPARα ligand, improved psoriatic lesions (40).

Further studies aimed at the identification of selective agonists and antagonists of the members of the PPAR family, and the potential of the biological role of methyl palmitate in the pancreas are needed.

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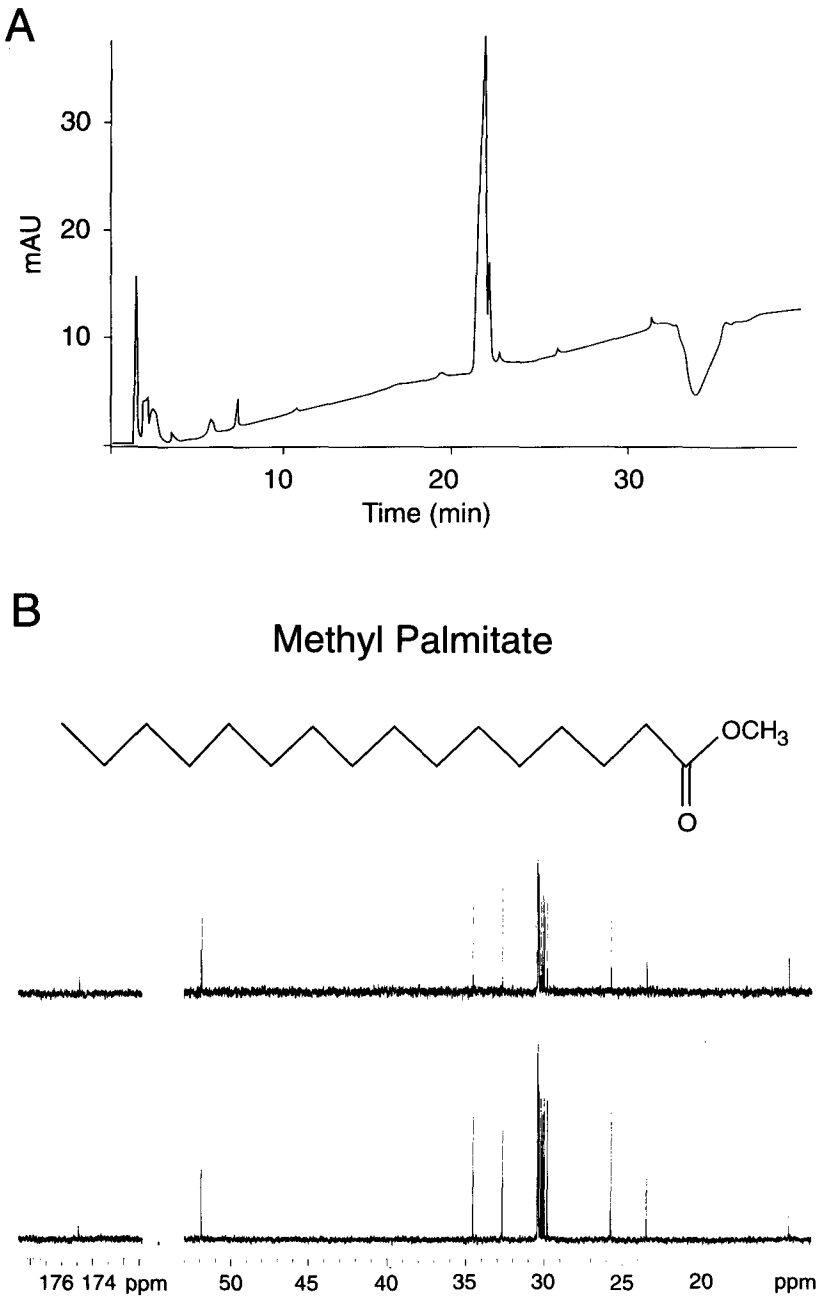


FIG. 5. Methyl ester of palmitic acid is the active component in pool 2. The purity of pool 2 of the C_4 HPLC column was assessed by analytical C_4 HPLC (A). Part B depicts the carbon nuclear magnetic resonance (NMR) spectra (in CD_3CN) of the purified pool 2 material (B, bottom) and of authentic sample of methyl ester of palmitic acid (B, top). The NMR data were: ^1H NMR (500 MHz, CD_3CN) δ 3.60 (*s*, 3H, OCH_3), 2.27 (*t*, 2H, $-CO-CH_2-$), 1.56 (*p*, 2H, $-CO-CH_2-CH_2-$), 1.28 (*m*, 24H), 0.88 (*t*, 3H, $-CH_2-CH_3$). ^{13}C NMR (125 MHz, CD_3CN) δ 174.92 (–O–CO–), 51.90 (OCH_3), 34.56, 32.71, 30.45, 30.44, 30.42, 30.42, 30.40, 30.35, 30.24, 30.14, 30.03, 29.83, 25.75, 23.46, 14.45. See Figure 3 for other abbreviation.

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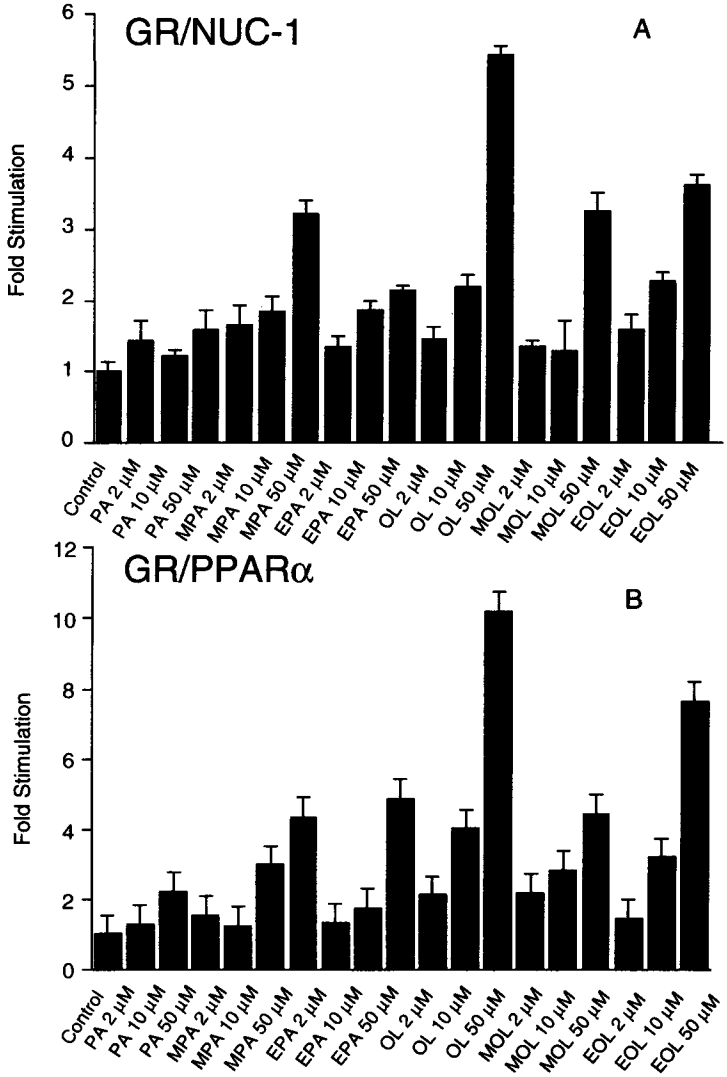


FIG. 6. Activation of GR/NUC-1 and GR/PPARα by free fatty acids and their methyl and ethyl esters. Commercially available palmitic (PA) and oleic (OL) acids and their methyl (M) and ethyl (E) esters were tested for the activation of GR/NUC-1 (A) and GR/PPARα (B) in ligand-dependent transcription assays at the indicated concentrations as described in the Materials and Methods section. Data are means + STD of fold stimulation relative to controls (dimethylsulfoxide).

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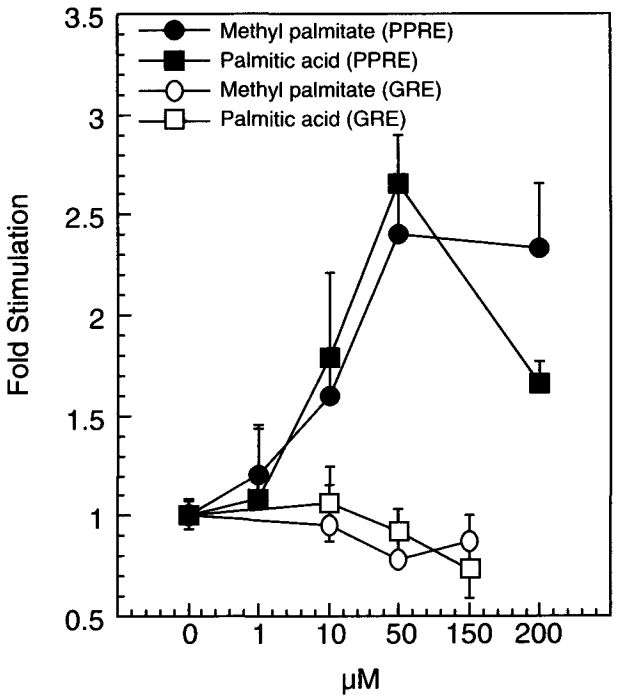


FIG. 7. Activation of native NUC-1 receptor by methyl palmitate. The native NUC-1 receptor was co-transfected with the MMTV-luciferase reporter gene that is under the control of the GRE or the PPRE hormone response element as described in the methods.

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Kinetics of Formation of Fluorescent Products from Hexanal and L-Lysine in a Two-Phase System

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ABSTRACT: Kinetics of formation of fluorescent condensation products from hexanal and L-lysine (or its *N*-acetylated forms) including mass-transfer has been studied in a two-phase system consisting of lysine (or lysine derivative) in an aqueous phosphate buffer and a 1-octanol solution of hexanal as model for formation of fluorophores between protein and carbonyl compounds in peroxidizing biological systems. The initial rate of formation of fluorescent products in the aqueous phase was found to be proportional to the concentration of hexanal and lysine and to increase in both phases with increasing pH in the aqueous phase, in contrast to a higher-order dependence on hexanal in the octanol phase. At pH = 6.8, the temperature dependence of the appearance of fluorescent products corresponds to apparent energies of activation of 63 kJ · mol^{-1} and 87 kJ · mol^{-1} in the aqueous phase and the octanol phase, respectively. Fluorescent condensation products appeared faster in the octanol phase. However, by a kinetic analysis, the fluorescent products were shown to be formed in the aqueous phase, corresponding to the lower energy of activation and to the simple second-order kinetics, and subsequently distributed between the aqueous phase and the octanol phase. L-Lysine reacted faster than N_α-acetyl-L-lysine which reacted faster than N_ε-acetyl-L-lysine. Using fluorescence quantum yields, determined to be $1.4 \cdot 10^{-2}$ in octanol and $8 \cdot 10^{-3}$ in water at pH 6.8, an apparent partition coefficient of 17 (octanol/water) was determined for the condensation product of L-lysine. The steady-state fluorescence in the octanol phase was attributed to two components with fluorescence lifetimes at 25°C of 0.7 ± 0.05 ns and 5.1 ± 0.2 ns, assigned to hexanal and the condensation product, respectively. The emission spectra were resolved in the two components using phase-sensitive detection, and the condensation product had emission maximum at 405 nm.

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During peroxidation of unsaturated fatty acids or lipids, various volatile carbonyl compounds are formed as the result of carbon-carbon cleavage of alkoxyl radicals formed by homolytic cleavage of hydroperoxides. These carbonyl compounds are able to react with amino groups (1), as those present in lysine residues in proteins, to yield fluorescent condensation products [cf. (2)], and fluorescence measurements have been demonstrated (3,4) to yield excellent indices of lipid peroxidation in biological materials (5). Fluorescence is also frequently used for the assessment of lipid oxidation in meat products where good correlation is found with the sensory impression of rancidity (6).

In order to mimic the conditions in the living cell and in food systems with aqueous phases containing the proteins and lipid phases in which the secondary lipid peroxidation products originate, a two-phase system was developed with a phosphate buffer as the aqueous phase with lysine derivatives as the reactive amine, and 1-octanol as the lipophilic phase, which with its similar polarity mimics the carbon chain of phospholipids (7). Hexanal, originating from breakdown of 13-hydroperoxy linoleic acid, 13-hydroperoxy γ-linolenic acid, and 15-hydroperoxy arachidonic acid (8), is the volatile compound found in the largest amounts in the headspace of peroxidizing tissue and was therefore used as a model for reactive carbonyl volatiles in the present study.

The reaction between lipid peroxidation products and amines is also of interest in relation to the *in vivo* formation of lipofuscin-like compounds during aging. The fluorescence of lipofuscin is not caused by a single fluorophore, as the colors of the emission range from blue to orange [cf. (9)]. Results from *in vitro* studies of formation of fluorophores related to lipofuscin, which notably showed only violet or blue fluorescence (10–12), have accordingly been questioned as being of relevance as lipofuscin models (13). A more detailed photophysical characterization of the involved fluorophores is not available. A comparison of the fluorophores formed *in vitro* and *in vivo* is expected to be more conclusive when based on fundamental photophysical parameters such as fluorescence quantum yields and emission lifetimes (14,15). Such information is also essential in order to distinguish between extracted fluorescent oxidation products and co-extracted fluorophores which are not related to lipid oxidation.

MATERIALS AND METHODS

Materials. 1,4-*Bis*(4-methyl-5-phenyl-oxazolyl)benzene (dimethyl-POPOP) scintillation grade was purchased from Fluka (Buchs, Switzerland). Rhodamine 101 laser grade was from Exciton (Dayton, OH). L-lysine monohydrochloride

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Abbreviation: Dimethyl-POPOP, 1,4-*bis*(4-methyl-5-phenyl-oxazolyl)benzene.

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biochemical grade and hexanal (>98% purity) was from Merck (Darmstadt, Germany), N_ε-acetyl-L-lysine and N_α-acetyl-L-lysine was from Sigma (St. Louis, MO). All other reagents were of analytical grade. Water was purified through a Millipore Milli-Q unit (Milford, MA), and only glassware was used for the experiments.

The pH was measured with a combination glass electrode (Ingold type 405) in conjugation with a Metrohm (Herisau, Switzerland) 605 pH meter. The electrode was standardized against titrated aqueous solutions of hydrochloric acid in 0.16 M NaCl, employing the definition pH = $-\log [H^+]$ throughout.

Two-phase model system. Hexanal was dissolved in 20 mL 1-octanol, and the L-lysine salt or derivative was dissolved in 50 mL 0.050 M aqueous phosphate buffer adjusted with NaCl to yield an ionic strength of 0.16. The aqueous phase was sterile filtered (0.22 μm filter) prior to use in order to prevent microbial growth. The two phases were constantly mixed in closed 300 mL gas-washing bottles by stirring with Teflon-coated magnetic stir bars in a thermostatted water bath.

Fluorescence measurements were carried out with an SLM 48000S multifrequency, phase-modulation spectrofluorometer (SLM Instruments Inc., Urbana, IL) with a 450-W Xe arc lamp, SLM MC200 excitation and MC320 emission monochromator mounted with Hamamatsu R928P photomultiplier tube at right angle to the line of illumination. Emission spectra reported were corrected for instrument sensitivity characteristics, and excitation spectra were corrected for lamp spectral characteristics by the method of ratio recording (16) using a solution of rhodamine 101 (3 g/L ethylene glycol) as quantum counter. Measurements were made in thermostatted quartz cells with right angle observation at the center. Air-saturated solutions were used for the measurements, except where otherwise noted. Fluorescence emission intensities were measured relative to an 0.10 mg/L solution of quinine sulphate in 0.50 M H_2SO_4.

Fluorescence quantum yield. The absorption of the products formed was measured in cuvettes with 50-mm light pathway in a Shimadzu UV-2101 PC spectrophotometer (Shimadzu, Duisburg, Germany). The fluorescence quantum yield (Φ_f) was determined by the optically dilute concept (17) using a solution of quinine sulfate in aqueous 0.5 M H_2SO_4 as fluorescence quantum yield reference [$\Phi_f = 0.546$ (18)] and correction for refractive indices [$n_{D,25}$(octanol) = 1.4276, $n_{D,25}$(water) = 1.3325; both values from (19)].

Emission lifetimes. The fluorescence lifetimes were determined by the multifrequency phase and modulation technique using an Omnichrome Series 2074 continuous wave He-Cd laser (Omnichrome, Chino, CA) as a light source (Schott UG11 325nm bandpass filter) in the SLM 48000S spectrofluorometer with the intensity of the exciting light sinusoidally modulated by means of a Pockel's cell. The instrument was set up at "magic angle" conditions, and emission was collected through a Schott KV 389-cut-on filter. Phase and modulation lifetimes (τ_p and τ_m) were calculated according to (20):

$$\tau_p = \frac{\tan\phi}{\omega} \qquad \tau_m = \frac{\sqrt{(1/m^2)-1}}{\omega} \qquad [1]$$

where ϕ is the phase shift in degrees, m the relative modulation, and ω the angular modulation frequency ($\omega = 2\pi \times$ frequency). Dimethyl-POPOP dissolved in absolute ethanol was used as a fluorescence lifetime standard [1.45 ns (21)]. At each frequency, the phase difference and demodulation were taken as the average of ten measurements.

Frequency-domain data analysis. As described in detail in Reference 22, calculated phase ($\phi_{c\omega}$) and modulation ($m_{c\omega}$) were estimated from the measured phase (ϕ_ω) and modulation (m_ω) values, the estimated uncertainties in the phase ($\sigma_{\phi\omega} = 0.5°$) and modulation ($\sigma_{m\omega} = 0.005$), and evaluated by the reduced chi-square χ^2_R:

$$\chi^2_R = \frac{\chi^2}{\nu} = \sum_\omega \frac{1}{\sigma^2_{\phi\omega}}(\phi_\omega - \phi_{c\omega})^2 + \sum_\omega \frac{1}{\sigma^2_{m\omega}}(m_\omega - m_{c\omega})^2 \qquad [2]$$

For the present analysis, we made measurements at 16 frequencies, with $\nu = 2(16) - 3 = 29$ degrees of freedom for fitting to a two-component decay (three repetitions).

Phase-sensitive detection of emission spectra. The steady-state emission spectrum, $F(\lambda,\phi_D)$, at detector phase, ϕ_D, from a sample containing two fluorophores, A and B with lifetimes $\tau_A < \tau_B$, using modulated light and phase-sensitive detection is (23)

$$F(\lambda,\phi_D) = F_A(\lambda)m_A\cos(\phi_D - \phi_A) + F_B(\lambda)m_B\cos(\phi_D - \phi_B) \qquad [3]$$

$F_A(\lambda)$ and $F_B(\lambda)$ are intensities of components A and B at wavelength λ, ϕ_A and ϕ_B are the phase angles, as calculated by Equation 1 using modulation frequency of 30 MHz. The steady-state spectrum of the unsupressed fluorophore was obtained by selecting the detector phase to be out of phase, $|\phi - \phi_D| = 90°$, with the emission of the other component.

RESULTS

Upon reaction in the two-phase system with hexanal dissolved in the octanol phase and L-lysine dissolved in the aqueous phase, a weak fluorescence was accumulating with a structureless emission spectrum in both phases with $\lambda_{em,max}$ at 420 nm for the aqueous phase (independent of excitation wavelength for 330–370 nm excitation) originating in a structureless excitation spectrum with maximum at 350 nm. The fluorophore formed was distributed between the aqueous phase and the octanol phase with $\lambda_{em,max}$ blue-shifted 5 nm (24) in the less polar octanol phase. The reaction was accompanied by the release of protons, as demonstrated in preliminary experiments with unbuffered solutions, but in experiments carried out in deoxygenated solutions (N_2-flushing), the rate of condensation was found not to depend on O_2 thus excluding hexanal oxidation as source of H^+, in accordance with Reference 25, and experiments were consequently performed with air-saturated solutions. The reaction order for the

reactants was determined using the method of initial rates [flooding of one of the reactants (26)]. In the pH region studied (5.3 to 6.8), as may be seen in Figure 1A for experiments with a constant concentration of L-lysine and varying hexanal concentration, and in Figure 1B for experiments with a constant concentration of hexanal and varying L-lysine concentration, the rate of formation of fluorescent products was in aqueous solution proportional to the concentration of both reactants at low concentrations in agreement with the overall rate law

$$\frac{dI}{dt} \propto \frac{d[\text{fluorophore}]}{dt} = k[\text{hexanal}][\text{L-lysine}] \qquad [4]$$

For both types of experiments, the rate of appearance was higher in the octanol phase compared to the aqueous phase, and in the experiment with (large) excess of hexanal, a significant curvature was noted for the appearance of fluorescence in the octanol phase. The results presented in Figure 1 are from the high-pH experiment (pH = 6.8), at lower pH the curvature was less significant (data not shown).

In order to investigate whether the higher rate observed for appearance of fluorescence in the octanol phase compared to the aqueous phase and the noted deviation from second-order reaction in the octanol phase was due to (i) a partitioning phenomenon, (ii) a lower fluorescence quantum yield in the aqueous phase compared to the octanol phase, or (iii) a faster rate of reaction in the octanol phase, the fluorescence quantum yield of the fluorophore(s) was determined in each phase. In a series of experiments at pH = 6.8 at 37°C with 1 M hexanal in the octanol phase, the fluorescence was followed in each phase for varying concentrations of L-lysine in the aqueous phase. As may be seen from Figure 2, the fluorescence intensity relative to a quinine standard reaches constant values in the aqueous phase after approximately 7 d for each L-lysine

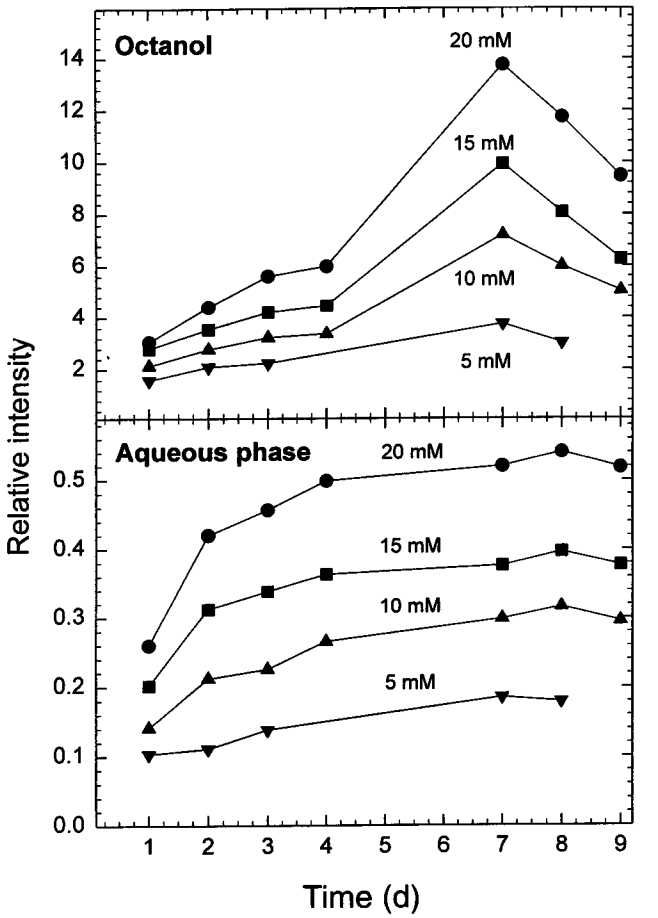


FIG. 2. Time course of formation of fluorescence in each phase of a two-phase system with 1 M hexanal in 1-octanol (20 mL) and varying concentration of L-lysine, as marked in the figure, in the aqueous phase (50 mL of 50 mM phosphate buffer, pH 6.8, $I = 0.16$) at 37.0 ± 0.5°C. Fluorescence intensity is measured relative to a 0.10 mg/L solution of quinine sulphate in 0.5 M H_2SO_4.

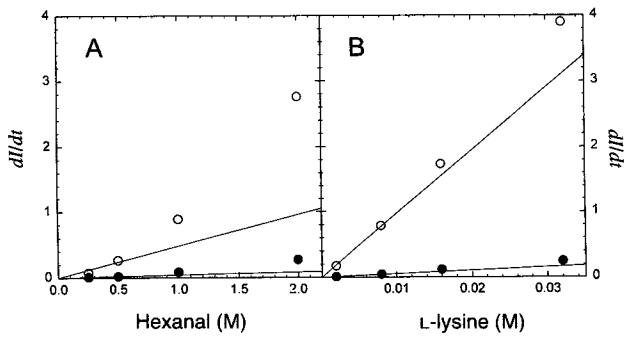


FIG. 1. Initial rate of formation of fluorescent products at 37.0 ± 0.5°C in the aqueous phase (●) and the octanol phase (○) of a two-phase system where *dI/dt* is the change in the relative intensity of emitted light at 410 nm with time for the first 2800 min of reaction. A, fixed concentration (10 mM) of L-lysine in the aqueous phase (50 mL of a 50 mM phosphate buffer, pH 6.86, $I = 0.16$) and varying concentrations of hexanal in the 1-octanol phase (20 mL). B, fixed concentration (1.0 M) of hexanal in the 1-octanol phase (20 mL) and varying concentrations of L-lysine in the aqueous phase (50 mL of a 50 mM phosphate buffer, pH 6.74, $I = 0.16$).

concentration. The relative fluorescence intensity is linearly dependent on the L-lysine concentration as may be seen in Figure 3, showing that a single fluorophore (or fluorophores in a constant ratio) are formed from L-lysine. From the fluorescence intensity and the absorbance of the solution for the 20 mM L-lysine solution measured in a 5-cm cell, the quantum yield was calculated (17) to yield $\Phi_{f,w} = 8 \cdot 10^{-3}$ (in water). The results were more complex for the formation of the fluorophore in octanol, since the intensity decreased after 7 d of reaction, suggesting further reactions. However, the maximum intensity obtained after 7 d in the octanol phase also depends linearly on the L-lysine concentration (Fig. 3), and applying a similar analysis as for the aqueous phase, yielded $\Phi_{f,o} = 1.4 \cdot 10^{-2}$ (in octanol). These results are lower limits since a slight turbidity developed in the solutions used for absorbance measurements. It should be noted that the quantum yields at the low concentrations employed inherently are corrected for partition reaction between the two phases, since the calculations are independent of the actual

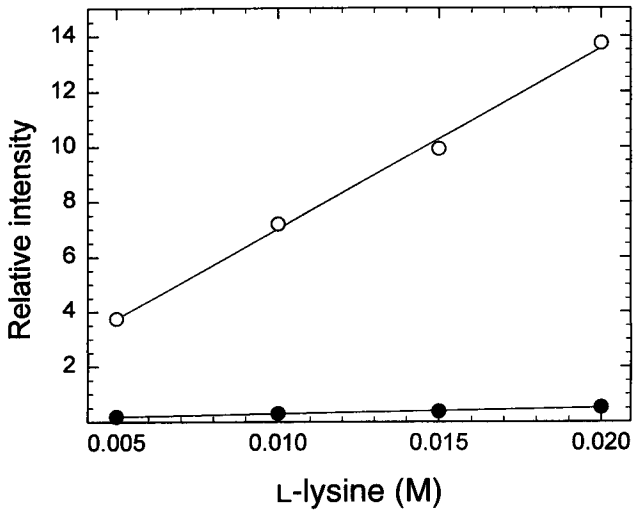


FIG. 3. Relative fluorescence intensity (measured relative to a 0.10 mg/L solution of quinine sulphate in 0.5 M H_2SO_4) after 7 d in aqueous phase (●) and octanol phase (○) for 1 M hexanal in octanol reacting with L-lysine in the aqueous phase, cf. Figure 2.

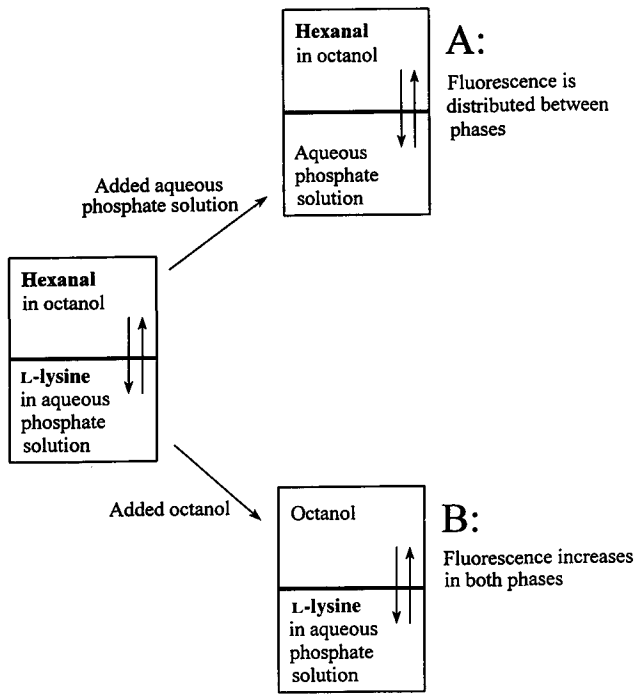


FIG. 4. Design of the reactant-removal experiment. After 7 d of reaction at 37.0 ± 0.5°C of a two-phase system with 20 mM of L-lysine in the aqueous phase (50 mL of a 50 mM phosphate buffer, pH 6.82, I = 0.16) and 1 M hexanal in the 1-octanol phase (20 mL), the phases were separated into two new bottles. The 1-octanol phase was transferred to bottle A and added the original volume of the pure phosphate buffer while the aqueous phase was transferred to bottle B and added the original volume of pure 1-octanol.

concentration of the fluorophore and only depend on the fluorescence intensity relative to the absolute quinine standard and on the absorbance. However, the fluorophore is distributed between the phases:

$$\text{fluorophore (aq)} \rightleftarrows \text{fluorophore (o)} \qquad [5]$$

for which the partition constant is defined and further related to the observed fluorescence intensity by:

$$K_p = \frac{[\text{fluorophore (o)}]}{[\text{fluorophore (aq)}]} \approx \frac{I_o}{I_w} \times \frac{\Phi_{f,w} \cdot \varepsilon_w \cdot n_w^2}{\Phi_{f,o} \cdot \varepsilon_o \cdot n_o^2} \qquad [6]$$

Accepting the further approximation, that the product of the molar absorptivity at the wavelength of excitation (ε) and the squares of the refractive index (n) is independent of the solvent, the experimental data (Fig. 3) yields K_p = [(14 − 1.8)/(0.52 − 0.1) × (8 · 10^{-3}/1.4 · 10^{-2})] = 17. The fluorophore is thus clearly more soluble in the lipohilic phase compared to the aqueous phase, suggesting that, if the fluorophore is formed in the aqueous phase, it is subsequently transferred to the octanol phase. In order to investigate the kinetics in further details, the following experiment, outlined in Figure 4, was designed: After 1 wk of reaction at 37°C in the two-phase system with 1 M hexanal in 20 mL of octanol and 20 mM L-lysine in 50 mL of aqueous phosphate buffer (0.050 M, pH 6.82, I = 0.16), the octanol phase was removed and mixed with pure phosphate buffer in another bottle (A), and the remaining aqueous phase was mixed with pure octanol (B). The fluorescence intensities of the two phases in the two separate bottles were then measured regularly during the next week. In both bottles the originally formed fluorophores were distibuted between the two phases within a few hours, but in contrast to bottle A, the fluorescence intensity increased in both phases of bottle B after the initial distribution of the fluorophores due to continued reaction between lysine and dissolved hexanal. Our findings are consistent with a mechanism where the aldehyde of the octanol phase dissolves in the aqueous phase in which the reaction takes place, and the fluorophores formed are subsequently distributed between the two phases (insert in Fig. 5). This mechanism is supported by the far higher solubility of hexanal in water [56 mM at 30°C (27), and a partition coefficient of $10^{1.78}$ (24)] than for lysine in octanol [0.08 mM, based on water solubility of 2.3 M and a partition coefficient of $10^{-4.44}$ (28)], and is in accordance with our observation of an interruption of fluorophore formation in the octanol phase of bottle A after removal of the aqueous phase containing lysine. The better linear dependence observed for the rate of appearance of fluorescence in the water phase on the concentration of excess of lysine (Fig. 1B), compared to the dependence for the rate of appearance of fluorescence in the octanol phase on the concentration of excess of hexanal (Fig. 1A), is in further support of this conclusion. However, the observed rates of appearance are only approximately 20 times larger [average value from Fig. 1 (A and B)] than in the aqueous phase, cf. Equation 4:

$$\frac{\left(\frac{dI}{dt}\right)_o}{\left(\frac{dI}{dt}\right)_w} = 20 = \frac{k_o[\text{hexanal}]_o[\text{lysine}]_o}{k_w[\text{hexanal}]_w[\text{lysine}]_w} \qquad [7]$$

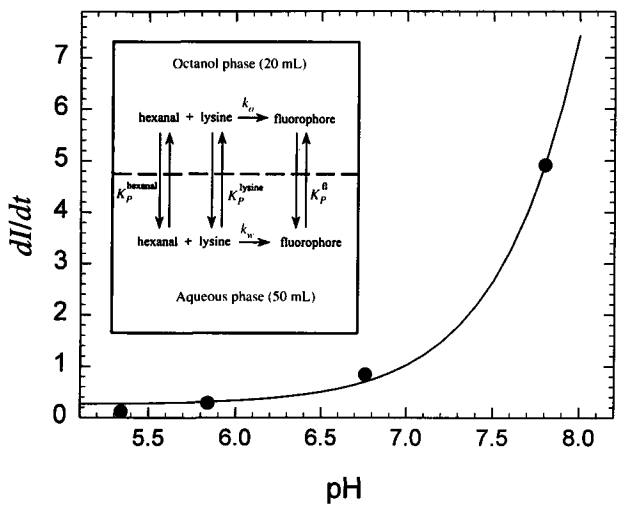


FIG. 5. Effect of pH in the aqueous phase on rate of formation of fluorescent products at 37.0 ± 0.5°C in the aqueous phase of a two-phase system consisting of 50 mL aqueous phase (10 mM L-lysine in 50 mM phosphate buffer with $I = 0.16$) and 20 mL octanol phase (1.0 M hexanal). *dI/dt* is the change in the relative intensity of emitted light at 410 nm with time for the first 2800 min of reaction. The full line is the best fit of the data to Equation 9 describing two parallel reactions of lysine in acid/base equilibrium with its protonated form (pK_a = 9.08 at 37°C and $I = 0.16$). Regression analysis yields $v_{R\text{-}NH_2} = (9.4 \pm 0.3) \cdot 10^{-5}$ and $v_{R\text{-}NH_3^+} = (2.5 \pm 0.7) \cdot 10^{-7}$ for the two relative rates, making the reaction of the protonated amine not significantly different from zero at a 5% level. Insert: kinetic model for the two-phase system.

and the ratio between the rate constant in octanol and in water accounting equally well for the observed appearance of fluorescence is:

$$\frac{k_o}{k_w} = \frac{dI_o}{dI_w} \times \frac{[\text{hexanal}]_w[\text{lysine}]_w}{[\text{hexanal}]_o[\text{lysine}]_o} = \frac{dI_o}{dI_w}\left(K_P^{\text{hexanal}} \cdot K_P^{\text{lysine}}\right)^{-1}$$

$$= 20 \cdot 10^{-1.78} \cdot 10^{4.44} = 9 \cdot 10^3 \qquad [8]$$

where K_p^{hexanal} and K_p^{lysine} are the partition coefficients between water and octanol for hexanal and lysine, respectively (24,27). It may thus be concluded that the rate constant for the octanol phase should be 9000 times the rate constant for the water phase to account equally well for the observed rate. The temperature dependence of the appearance of fluorescent products analyzed according to the Arrhenius equation for both phases yields further information. For the three temperatures investigated at pH 6.68 ± 0.05, a linear plot was observed both for the aqueous phase ($r^2 = 0.999$) and for the octanol phase ($r^2 = 0.998$), and apparent energies of activation $E_a = 63 \pm 2$ kJ · mol^{-1} for the aqueous phase and $E_a = 88 \pm 4$ kJ · mol^{-1} for the octanol phase were calculated. It appears unlikely that the significantly larger rate constant required to account for the reaction in the octanol phase compared to the aqueous phase has a significantly higher energy of activation.

The rate of reaction in both phases was found to be very dependent on the pH of the aqueous solution, and as shown in Figure 5, the rate of reaction increased more than an order of magnitude, when solution pH increases in the biologically relevant interval from 5.35 to 7.80. The rate of appearance of fluorescence at different pH was analyzed according to

$$\frac{dI}{dt} = \frac{[H^+]}{[H^+]+K_a} \times v_{R\text{-}NH_3^+} + \frac{K_a}{[H^+]+K_a} \times v_{R\text{-}NH_2} \qquad [9]$$

in agreement with parallel reactions in the aqueous phase of the protonated form of lysine (rate $v_{R\text{-}NH_3^+}$) and the non-protonated form of lysine (rate v_{NH_2}) in (rapid) equilibrium with each other with an acid dissociation constant, K_a, of $8.31 \cdot 10^{-10}$ (28). The reaction of the protonated form was by regression analysis found to be nonsignificant, and it can therefore be concluded that the condensation is a reaction between the free amino group of lysine and hexanal in the aqueous phase.

The relative reactivity of the two amino groups of lysine was studied by a comparison of the rate of reaction of the *N*-acetylated forms of lysine. The rate of reaction was found to be in the order L-lysine > N_α-acetyl-L-lysine > N_ε-acetyl-L-lysine, and the rate of formation of condensation products was highest in the octanol phase for all three forms of lysine (Fig. 6). The results show that hexanal is capable of reacting with both the ε-amino groups in proteins as well as the *N*-terminal amino group. Moreover, the higher reactivity of N_α-acetyl-L-lysine compared to N_ε-acetyl-L-lysine suggests that steric factors are important, since pK_a is 9.08 for the ε-amino group and 10.69 for the α-amino group (29). As may be seen from Figure 6, the octanol phase is slightly fluorescent prior to reaction. Hexanal is very weakly absorbing having ε = 4 M^{-1}cm^{-1} in 1-octanol at the absorption maximum 288 nm due to a symmetry-forbidden n→π* transition (30). The fluo-

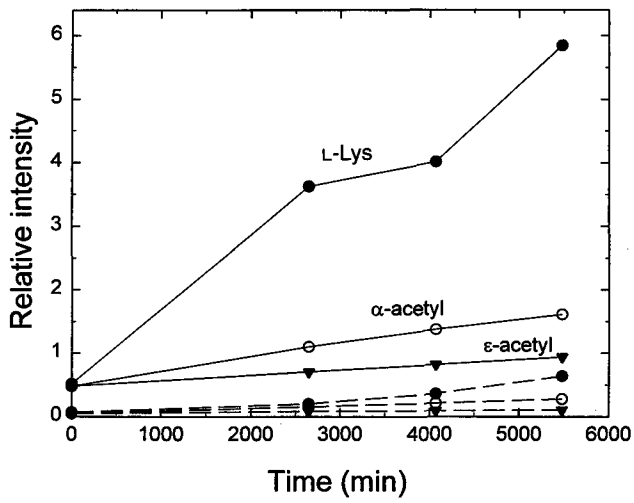


FIG. 6. Time course of formation of fluorescent products in the aqueous phase (broken line) and octanol phase (solid line) of a two-phase system consisting of 50 mL aqueous phase (10 mM of L-lysine (●), N_α-acetyl-L-lysine (○) and N_ε-acetyl-L-lysine (▼), respectively, in 50 mM phosphate buffer with pH = 6.86, $I = 0.16$) and 20 mL octanol phase (1.0 M hexanal). Emission intensity at 410 nm measured relatively to a 0.10 mg/L solution of quinine sulphate in 0.50 M H_2SO_4.

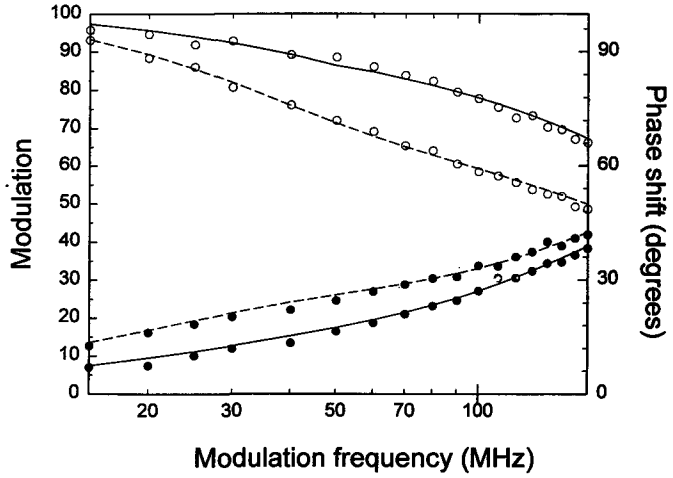


FIG. 7. Multifrequency phase and modulation data for the octanol phase of the reaction mixture (50 mL of 20 mM L-lysine in 50 mM aqueous phosphate buffer, $I = 0.16$, pH 6.83, and 20 mL of 1.0 M hexanal in 1-octanol), at 37.0 ± 0.5°C after 23 h of reaction (solid line) and 71 h of reaction (dashed line). The lines are a common best fit to the experimental data for both reaction times (●, measured phase shift; ○, modulation) using Equation 1 and Equation 2 with two exponential components with $\tau_1 = 0.7 \pm 0.05$ ns and $\tau_2 = 5.1 \pm 0.2$ ns, respectively.

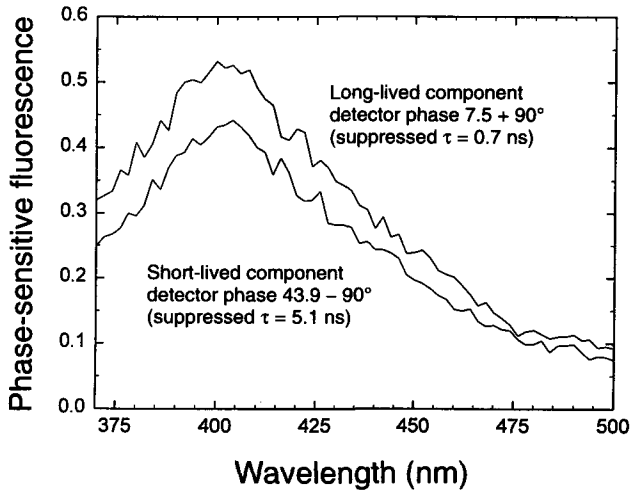


FIG. 8. Phase-sensitive emission spectra for the two components in the octanol phase of the reaction mixture described in legend to Figure 7, using 30 MHz modulation frequency.

rescence quantum yield is very low [$7 \cdot 10^{-4}$ in cyclohexane (31)]. In order to resolve the background fluorescence of hexanal from the condensation product, the phase and demodulation technique was used. Multifrequency phase and modulation data were best fitted to a bi-exponential decay function, and addition of a third component was not statistically justified. The fit yielded two fluorophores with lifetimes 0.7 and 5.1 ns, see Figure 7 for day 1 and 3. From the increased phase shift and lower modulation at all modulation frequencies, a larger contribution from the long-lived component to the fluorescence decay constant was found with time (fraction of 0.20 after 1 h of reaction to 0.48 after 123 h), and this component was accordingly assigned as the condensation product. Based on the two fluorescence lifetimes, the steady-state emission spectra were resolved using phase-sensitive detection. The similarity of these spectra (Fig. 8, for day 4 at pH 6.83) suggests that the fluorophore of the condensation product(s) is closely related to the parent carbonyl compound, as further evidenced by the partition coefficient.

DISCUSSION

In a two-phase model system with mass-transfer, we have shown that monofunctional aldehydes are able to react with L-lysine to yield fluorescent compounds under conditions of relevance for biological systems, as previously demonstrated by Kikugawa and Sawamura (32) in a solubilized system. Alkanals also react with proteins, and hexanal has been shown to react with polylysine (33), with erythrocyte ghosts (34), and eye lens α-crystallin (35), to yield fluorescent protein cross-links. Suyama and Adachi (36) proposed that the reaction proceeds through an α,β-unsaturated aldimine, which could be formed as an intermediate in amine-catalyzed aldol condensation or condensation of α,β-unsaturated aldehydes with amines. The formation of both 2-ethyl-2-hexenal and a tetrameric dialdehyde during reaction between butanal and benzylamine in a solubilized system (37) is further of support for this mechanism.

The demonstrated increase in rate of formation of fluorophores with increased pH is in accordance with the results of Fukuzawa *et al.* (38) on the reaction of 12-keto-oleic acid with glycine in a system consisting of 20% ethanol and 0.1 M phosphate buffer. It may thus be speculated that reaction depends on an initial aldol condensation to form an α,β-unsaturated aldehyde together with a deprotonization of the amino group to yield a stronger nucleophile. The aldol condensation has been shown to be catalyzed by amino acids or by phosphatidylethanolamine (36). The demonstrated partitioning of the alkanals between water and octanol, corresponding to partioning between membrane and serum, suggests a mechanism, in which peroxidation of the phospholipids in membranes yield alkanals, which are converted to α,β-unsaturated aldehydes in the lipid/water interface catalyzed by phosphatidylethanolamine in the membranes, after which these are able to react with available amino groups to form fluorescent condensation products. In this context, it is interesting to note that the fluorescent condensation product has a partition coefficient of 17, which is more similar to the partition coefficient of hexanal than lysine. Hexanal reacts with both the α-amino group, corresponding to the *N*-terminal of proteins, and the ε-amino group of lysine, and since the rates of formation of fluorescent products are not very different, it may be concluded that the ε-amino group plays the major role in the reaction of proteins due to the higher number of these groups. Moreover, alkanals constitute an important source for formation of α,β-unsaturated aldehydes also capable of reaction with sulphydryl groups of proteins (39), resulting in forma-

tion of a thioether linkage from a protein to C-3 of the aldehyde. The unreacted carbonyl group of this conjugate could further react with an amino group forming an unconjugated imine resulting in severe protein damage through a nonfluorescent cysteine-lysine cross-link. In fact, the high reactivity of α,β-unsaturated carbonyls toward thiols, such as glutathione and *N*-acetyl-cysteine, has been demonstrated to block the formation of fluorescent conjugates in mixtures with glycine and 12-keto-oleic acid giving only rise to nonfluorescent products (38). Although less reactive than alkenals and 4-hydroxy-alkenals, the importance of alkanals in the formation of fluorescent products and protein cross-links cannot be neglected as they are formed in significantly higher concentrations during the course of peroxidation of unsaturated lipids.

In conclusion, the present study has shown that the rate of formation of fluorophores from amino groups and aldehydes increases with pH and that the condensation is taking place in the serum phase and that fluorophores subsequently are "extracted" back to the lipid phase, indicating that the formation of blue fluorescent extractables would be tissue-dependent. The fluorescence of extracts is consequently expected to be very heterogeneous, and a complete understanding of the nature of extractable fluorescent lipid oxidation products and lipofuscin fluorescence will depend on a photophysical characterization of the fluorophores involved. When future research has provided this knowledge, it would be possible to analyze for specific fluorophores originating in oxidative transformations and thereby significantly improve the specificity of the fluorescence assay for evaluation of oxidative status.

ACKNOWLEDGMENTS

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Hydrolysis of Used Frying Palm Olein and Sunflower Oil Catalyzed by Porcine Pancreatic Lipase

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ABSTRACT: The enzymatic hydrolysis of frying used vegetable oils with different degrees of alteration were measured using porcine pancreatic lipase (acylglycerol acylhydrolase EC 3.1.1.3). Successive frying of potatoes significantly increased the level of total polar lipid content in the palm olein from 9.3 ± 0.1 mg/100 mg oil to 26.4 ± 0.3 mg/100 mg oil after 90 fryings, and from 4.0 ± 0.1 mg/100 mg oil to 27.7 ± 0.3 mg/100 mg oil in sunflower oil after 60 fryings. Triacylglycerol polymers, triacylglycerol dimers, and oxidized triacylglycerols also increased 37-, 7.9-, and 7.5-times in palm olein, respectively, and 56-, 22-, and 4.7-times in sunflower oil, respectively. However, diacylglycerols and free fatty acid levels related to hydrolytic alteration did not increase with the number of fryings in both oils. The substrate concentration in the reactor was determined by calculating the molecular weight of each oil showing a different degree of alteration. We compared the methodology used by us and that used by other authors. The results show that the methods are reproducible and that the values obtained are in concordance with theoretical values. The kinetic parameters apparent Michaelis-Menten constant (K_M^{app}) and apparent maximum velocity of hydrolysis (V_{max}^{app}) were different in unused palm olein (5.1 ± 0.7 and 166 ± 7.6, respectively) than in sunflower oil (2.2 ± 0.3 and 62 ± 2.2, respectively). However, changes in K_M^{app} and V_{max}^{app} were not related to the degree of alteration of the oils.
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Pancreatic lipase—triacylglycerol acylhydrolase (EC 3.1.1.3)—is a key enzyme for the intraluminar digestion of dietary fats. It hydrolyzes long-chain triacylglycerol derivatives into more polar fragments, mainly monoacylglycerols and free fatty acids, which are subsequently absorbed (1,2).

This enzyme shows fatty acyl specificities for chainlength, position, and geometry of double bonds (3). However, little is known about the kinetic behavior of pancreatic lipase toward the thermoxidized fatty acyl components in triacylglycerol molecules.

Deep-fat frying is an important method for preparing food that has become more common in the past few years. Nevertheless, during deep-fat frying the fat employed for this purpose is exposed to agents that cause drastic structural changes in it. Moisture from foodstuffs causes hydrolytic alteration; atmospheric oxygen entering the oil gives rise to oxidation, and the temperature (180°C) at which the operations take place accelerates chemical changes involving the formation of complex reaction products such as triacylglycerol polymers, triacylglycerol dimers, and oxidized triacylglycerols (4–7).

In order to know whether the digestion process of culinary fats is influenced by these collateral products developed during frying, it would be interesting to study whether pancreatic lipase activity is affected by the presence of these alteration products.

Edible oils which have been heated in the presence of air are well known to have less nutritive value than the corresponding fresh oils (8,9). This negative effect has been attributed to the presence of polymers. However, López-Varela *et al.* (10) have shown a similar fat absorption in rats fed a diet containing a high level of thermoxidized sunflower oil than in rats fed the unused oil. At present, it is not well known whether the lower absorption of heated fat is due to deficiencies in the step of enzymatic hydrolysis to reduce molecular weight (MW) of complex glycerides or to difficulties in the absorption of complex fatty acids released by the action of pancreatic lipase. Results obtained on oxidized triacylglycerol monomers indicate that hydroperoxides undergo hydrolysis to the same extent as normal triacylglycerols (11). However, results of Márquez Ruiz *et al.* (12) clearly demonstrate that enzymatic hydrolysis depends on the MW of the glycerides being slower as alteration compound MW increases.

In order to know whether the digestion process of culinary fats is influenced by these collateral products developed during frying, it would be interesting to study whether pancreatic lipase activity is affected by the presence of these alteration products.

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Abbreviations: ANOVA, analysis of variance; FID, flame-ionization detector; HPLC, high-performance liquid chromatography; HPSEC, high-performance size exclusion chromatography; K_M^{app}, apparent Michaelis-Menten constant; MW, molecular weight; PEG, polyethylene glycol; r_t, retention time; THF, tetrahydrofuran; V_{max}^{app}, apparent maximum velocity of hydrolysis.

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Recently, a marked increase in the consumption of palm olein (containing approximately 43% oleate) for frying purposes has been described in Mediterranean countries (13). Economic reasons and the high oxidative stability of palm olein, as described by Gupta (14), are responsible for the use of palm olein for frying and for snack foods.

As far as we know, no work has been done studying the kinetic parameters of thermoxidized palm olein. For this reason, the aims of the present work were (i) to study the thermoxidative alteration of palm olein and sunflower oil (two culinary fats that differ in their fatty acid composition) and (ii) to determine the kinetics of the enzymatic hydrolysis of used frying vegetable oils with different degrees of alteration by porcine pancreatic lipase *in vitro* in order to elucidate the effect of the complex reaction products arising during frying on lipolysis.

MATERIALS AND METHODS

Palm olein was provided by AGRA S.A. (Bilbao, Spain). Refined sunflower oil Koipesol (Córdoba, Spain) and potatoes (Kennebec variety; Galicia, Spain) were purchased at a local store. Silica gel 60 F_{250} (63–200 μm) for column chromatography and silica gel 60 (20 × 20 cm, 0.2 mm layer thickness) for thin-layer chromatography were obtained from E. Merck (Darmstadt, Germany). Triolein, diolein, monoolein, oleic acid, and lipase (EC 3.1.1.3) from porcine pancreas (Type II) were obtained from Sigma Chemical Co. (St. Louis, MO). Polyethylene glycols of different MW (3000, 1500, 1000, 400, and 200) were obtained from Fluka Chemie AG (Buchs, Switzerland). All other chemicals used were of the purest degree available. Aqueous solutions were made with double-distilled water.

Frying performances. The deep-fat frying operations were carried out in domestic electric fryers that contained 3 L of oil. The oil was heated to 180°C (20 min), and then 500 g of potatoes (slices *ca.* 2 mm thick) were fried for 8 min. A total of 90 fryings with palm olein and 60 fryings with sunflower oil, at a rate of 10 fryings per day were carried out. The overall time that the oils were heated throughout the whole experimental period can be estimated as 30 h, in the case of palm olein, and 20 h for sunflower oil. No fresh oil was added at any time. Extensive details of the frying method and the temperature changes have been described previously (7).

Aliquots of 50 mL of the unused oil and the corresponding used oil from different fryings were taken for analysis. All samples were kept at –20°C under an atmosphere of nitrogen until analysis.

Analytical determinations. (i) *Determination of the fatty acid composition of unused oils.* Unused palm olein and sunflower oil were saponified with 0.5 N sodium hydroxide and then methylated following the method suggested by the IUPAC (15). The fatty acid methyl esters of the oils were then analyzed by gas chromatography on a Hewlett-Packard (Palo Alto, CA) 5890 Series II chromatograph fitted with a flame-ionization detector (FID). Analyses were effected on a steel column packed with 10% Supelcoport 2330 (Supelco, Bellefonte, PA) on 100/120 Chromosorb WAW 2 m × 1/8 in. The temperature of the column was held for 8 min at 170°C and then increased to 240°C at 2°C/min. The temperature of the injector was 250°C and that of the detector 280°C. Sample size was 1 μL. The peak areas were measured using a Hewlett-Packard 3396 Series II integrator.

(ii) Oil sample analysis. Oil samples were analyzed throughout the frying operations by a combination of adsorption and size exclusion chromatography, which includes: (a) determination of polar compounds by silica column chromatography, following the method of Waltking and Wessels (16), using a modified proportion of hexane/diethyl ether to fill the column and to elute the nonpolar fraction (7). The efficacy of the separation was then checked by thin-layer chromatography to guarantee the absence of unaltered triacylglycerols in the polar lipid fraction. (b) Distribution of polar compounds by high-performance size-exclusion chromatography (HPSEC). Following the method of Dobarganes *et al.* (6), polar compounds were analyzed in a Waters 501 chromatograph (Milford, MA) with a 20 μL sample loop. A Waters 410 refractive index detector and two 0.01 and 0.05 μm (5 μm particle size) pL gel (polystyrene–divinylbenzene) columns (7.5 × 300 mm) (Hewlett-Packard) connected in series and operated at 40°C were used. High-performance liquid chromatography (HPLC)-grade tetrahydrofuran (THF) served as the mobile phase with a flow of 1 mL/min. Sample concentrations were between 10 and 15 mg/mL in THF. All eluents and samples were precleaned by passing them through a 2 μm filter. The quantity of each compound was calculated as previously described (17).

Determination of average MW of oils. The average MW of unused oils and of the corresponding oils used in different fryings were calculated by a calibration curve using HPSEC (18), which is a valuable technique in providing information about sample complexity, accurate MW, and their distribution patterns. In a size exclusion chromatography column, the analytes are eluted in accordance with their molecular sizes, i.e., hydrodynamic volume in a mobile phase solvent.

HPSEC retention times of standard acylglycerides (triolein, diolein, monoolein, and oleic acid) on two 0.01 and 0.05 μm (5 μm particle size) pL gel (polystyrene–divinylbenzene) (Hewlett-Packard) columns were determined by injecting individual samples with THF as the eluent at a flow rate of 1 mL/min. Calibration curves were fitted with the SIMFIT statistical package (19).

Sample preparation for enzyme assays. The substrate was prepared by emulsifying 7.5 mL oil (palm olein or sunflower oil) and 7.5 mL Tris-HCl buffer 0.01 M (pH 8.3) with 35 mL of emulsifying reagent (20) using an ultrasonic generator (sonifier 450; Branson Ultrasonics Corp., Danbury, CT) equipped with a 10-mm probe. The probe was placed in the bulk of the solution to be emulsified and its output activated at 80 watts for 3 min. The preparation was then left to stand for 1 min. This operation was repeated twice. The emulsifying reagent (1 L) contained glycerol (540 mL), arabic gum

(10 g), NaCl (17.9 g), KH_2PO_4 (0.41 g) and twice distilled water.

Enzyme assays. Titrimetric assay was employed to determine lipolytic activity (21). The acid released in the hydrolysis was continuously titrated at pH 8.3 with the aid of a Metrohm Ltd. pH-stat [Impulsomat 614, Dosimat 665 (with microstirrer) and pH-meter 691] (Herisau, Switzerland). Several NaOH solutions (5 to 50 mM) were used as titrating agents.

The reaction mixture consisted of different amounts of substrate emulsion (10–150 μL), 1 mL of lipase solution (10 mg/mL), and 0.01 M Tris-HCl buffer (pH 8.3) up to 10 mL total volume. In a typical experiment, Tris-HCl buffer (pH 8.3) and substrate emulsion were placed in the 37°C-thermostated cuvette of the pH-stat. The mixture was shaken vigorously for 10 min at 37°C. Stirring rate was adjusted to approximately 1300 rpm. Then, 1 mL of lipase solution (10 mg/mL) in Tris-HCl buffer (pH 8.3) was added. The reaction time was 10 min.

In all cases, lipase activity was measured as initial reaction rates in order to avoid the possible inhibition that might take place due to the appearance of reaction products. The slopes of the initial linear stretch of the kinetic curves were determined graphically. Experiments were performed in triplicate, and relative standard errors were less than 5%. Specific lipase activity was defined as the μmol of free fatty acids released per minute and per mg of crude enzyme.

Statistical analysis. Data from the different oils were compared by one-way analysis of variance of repeated measures, followed by multiple-range analysis.

Curve fitting and statistical treatment of the data from enzyme assays were performed using the SIMFIT package (19). Kinetic behaviour of used and unused oils were considered not statistically different when their confidence limits are to a certain extent overlayed.

RESULTS AND DISCUSSION

Composition of unused and repeatedly fried oils. The total polar lipid contents from palm olein and sunflower oil are shown in Table 1, as a representative measurement of the total alteration in the oils together with the contents of different groups of altered products. In both cases, the results were obtained from unused oil and the corresponding oil used in different fryings.

As may be seen in Table 1, the polar lipid content of used palm olein showed a linear increase throughout the successive fryings of potatoes from 9.3 ± 0.1 mg/100 mg unused oil to 26.4 ± 0.3 mg/100 mg oil after ninety fryings. According to Fedeli (22), the speed of degradation is proportional to temperature and the frying time. Accordingly, the total polar lipid content of sunflower oil increased during frying from 4.0 ± 0.2 mg/100 mg unused oil to 27.7 ± 0.3 mg/100 mg oil after sixty fryings (Table 2).

When the alteration levels produced by frying in both cooking oils were compared (Table 1), despite the higher percentage of total polar lipid content in unused palm olein with respect to fresh sunflower oil, a higher increase in the total alteration percentage was observed in sunflower oil as compared to palm olein (60 fryings vs. 90 fryings, respectively) for a similar polar content to be reached (approximately 27%).

Sunflower oil has a higher unsaturated fatty acid content than palm olein, as may be observed in Table 2. These results are in agreement with those reported by other authors (23).

In this sense the lower alteration in palm olein as compared to sunflower oil after a similar number of fryings, in accordance with several authors (24–26), indicates that alterations in used frying fats are related to their degree of unsaturation as well as the heat treatment. The results show that palm olein exhibits a unique stability in frying applications.

The polar fraction of unused and used palm olein was further examined by HPSEC to investigate thermooxidative and hydrolytic alterations in the frying oil.

Quantitation of the different groups of alteration compounds in palm olein and sunflower oil, respectively, is shown in Table 1. As can be seen, triacylglycerol polymers, triacylglycerol dimers and oxidized triacylglycerols, which involve thermooxidative alteration, increased throughout the fryings in both culinary oils, reaching higher contents in sunflower

TABLE 1
Groups of Compounds in Unused Palm Olein and in Oil Samples After Being Used in Repeated Fryings of Potatoes[a]

				Thermooxidative alteration			Hydrolytic alteration		
Oil	Number of fryings	Nonpolar TG	Total polar content	TG polymers	TG dimers	Oxidized TG	DG	MG	FFA
Palm olein	0	90.7 ± 0.1[a]	9.3 ± 0.1[a]	0.1 ± 0.0[a]	1.0 ± 0.3[a]	1.1 ± 0.2[a]	6.7 ± 0.2[a]	0.04 ± 0.0[a]	0.2 ± 0.1[a]
	60	81.8 ± 0.2[b]	18.2 ± 0.2[b]	1.6 ± 0.0[b]	5.0 ± 0.1[b]	5.3 ± 0.1[b]	6.1 ± 0.1[a]	0.04 ± 0.0[a]	0.2 ± 0.0[a]
	90	73.6 ± 0.3[c]	26.4 ± 0.3[c]	3.7 ± 0.0[c]	7.9 ± 0.1[c]	8.3 ± 0.1[c]	6.2 ± 0.1[a]	0.04 ± 0.0[a]	0.2 ± 0.0[a]
	ANOVA	<0.001	<0.001	<0.001	<0.001	<0.001	NS	NS	NS
Sunflower	0	96.0 ± 0.2[a]	4.0 ± 0.2[a]	0.1 ± 0.0[a]	0.5 ± 0.0[a]	1.9 ± 0.1[a]	1.0 ± 0.1[a]	—	0.5 ± 0.0[a]
	30	81.1 ± 0.3[b]	18.9 ± 0.3[b]	2.2 ± 0.1[b]	7.8 ± 0.2[b]	7.0 ± 0.2[b]	1.4 ± 0.1[b]	—	0.5 ± 0.0[a]
	60	72.3 ± 0.3[c]	27.7 ± 0.3[c]	5.6 ± 0.2[c]	11.0 ± 0.2[c]	8.9 ± 0.2[c]	1.7 ± 0.1[b]	—	0.5 ± 0.1[a]
	ANOVA	<0.001	<0.001	<0.001	<0.001	<0.001	<0.01	—	NS

[a]Values are means of three samples ± SD expressed in mg/100 mg oil. Values in the same column bearing a common letter are not significantly different. TG, triacylglycerols; DG, diacylglycerols; MG, monoacylglycerols; FFA, free fatty acids; ANOVA, analysis of variance; NS, not significant.

TABLE 2
Major Fatty Acid Composition (%) of Unused Palm Olein and Sunflower Oil[a]

Fatty acids	Palm olein	Sunflower oil
$C_{14:0}$	1.10 ± 0.04	—
$C_{16:0}$	38.60 ± 0.40	6.76 ± 0.02
$C_{18:0}$	4.02 ± 0.01	3.71 ± 0.07
$C_{18:1}$	44.90 ± 0.20	32.52 ± 0.04
$C_{18:2}$	10.30 ± 0.40	55.55 ± 0.09

[a]Mean of three samples ± SD.

oil. This is due to the higher content of unsaturated fatty acids in sunflower oil as compared with palm olein (see Table 2) and it is responsible for higher stability in palm olein when heated or oxidized.

Furthermore, hydrolytic reactions did not increase during consecutive fryings of potatoes in palm olein as shown in Table 1, because diacylglycerols, monoacylglycerols, and free fatty acids remained at similar levels throughout the fryings. However, the diacylglycerol content in sunflower oil increased slightly; by contrast, free fatty acid contents remained unchanged throughout the successive fryings (Table 1).

The above results clearly indicate that in deep-fat frying of potatoes thermooxidative reactions take place preferentially over hydrolytic reactions. It can be also concluded that, in general, discontinuous and successive fryings of potatoes increase the degree of polymerization of culinary fats. These results are in agreement with those reported by several authors (27,28).

Average MW of unused and repeatedly heated frying oils. In order to carry out a study on the pancreatic lipase activity in several unused and used frying oils, we needed to establish in some way the substrate concentration in the reactor. We therefore addressed the problem of calculating the MW of each unused and fried oils showing a different degree of alteration. The method used was to perform calibration curves with standards on HPSEC.

The calibration curves of retention times (r_t) vs. log MW are shown in Figure 1.

The corresponding equation for standard acylglycerols was:

$$\log MW = 6.87 - 0.30\, r_t \qquad [1]$$

with a correlation coefficient of $r = -0.998$. However, the lack of acylglycerol standards with a higher MW than triacylglycerols (>900) prevents the use of this equation to calculate the MW of the compounds with different degrees of polymerization after frying. Consequently, a calibration curve (Fig. 1) was made up using polyethylene glycol (PEG) standards of various MW (3000, 1500, 1000, 400, and 200) (18). The corresponding equation was:

$$\log MW = 6.53 - 0.26\, r_t \qquad [2]$$

with a correlation coefficient of $r = -0.999$. When this calibration Equation 2 was applied to confirm the MW of the acylglycerol standards, significant differences were observed; these were higher than the expected values. This suggests that a certain interaction must take place between the mobile phase and the acylglycerols (18). Therefore, Equation 2 may not be applied when calculating the MW of acylglycerols due to their different chemical properties with respect to PEG, which induces slight differences in their r_t. This was demonstrated by statistically comparing Equation 1 and Equation 2 which proved to be significantly different (ANOVA of regression coefficient; $P < 0.001$). Accordingly, calculation of the MW of the different compounds with different degrees of polymerization by extrapolation to Equation 1 affords a lower error than Equation 2 for the above indicated reasons. Equation 1 was therefore applied to calculate the MW of the compounds of the thermooxidized oils.

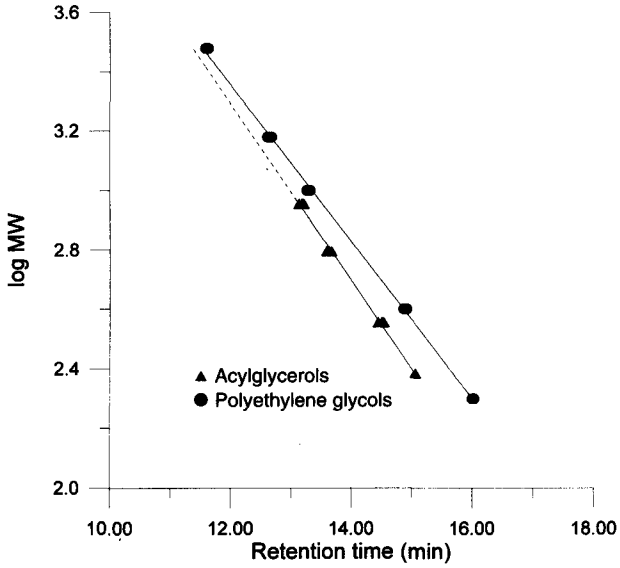


FIG. 1. Determination of molecular weights (MW) of oils. Calibration graph using acylglycerols (▲) and polyethylene glycols (●) as standards. (—) corresponds to computer fitting of the data to linear equation. (---) corresponds to the estimated linear equation for acylglycerols with MW between 900 and 3000.

Table 3 gives the estimated average MW of unused palm olein and sunflower oil as 855 and 865, respectively. Likewise, Table 3 offers the estimated average MW of each of the different samples of palm olein and sunflower oil after frying employed as substrates for porcine pancreatic lipase. As can be seen in this table, the average MW of culinary fats increased throughout the different fryings. This might be related to the formation of high MW compounds, such as triacylglycerol dimers and polymers.

Activity of pancreatic lipase against unused and repeatedly fried oils. To establish whether the alteration products arising during the frying process might affect pancreatic lipase activity among its natural substrates, enzymatic activity was measured in sunflower oil and palm olein from different fryings. In an attempt to quantify whether the kinetic behav-

TABLE 3
Average Molecular Weights of Palm Olein and Sunflower Oil, Unused and After Fryings, Determined by High-Performance Size-Exclusion Chromatography

	Number of fryings	Average molecular weight
Palm olein	0	855
	60	905
	90	951
Sunflower oil	0	865
	30	943
	60	1005

ior is influenced by frying, $v - [S]$ studies where $v - [S] = v$ vs. $[S]$; where v is the initial velocity in μmol/min · mg enzyme and $[S]$ is substrate concentration is mM were performed and the corresponding kinetic parameters were calculated. As an example, the corresponding study to palm olein and sunflower oil after sixty and thirty fryings both showing a similar degree of alteration [around 18% (Table 1)] is shown in Figure 2.

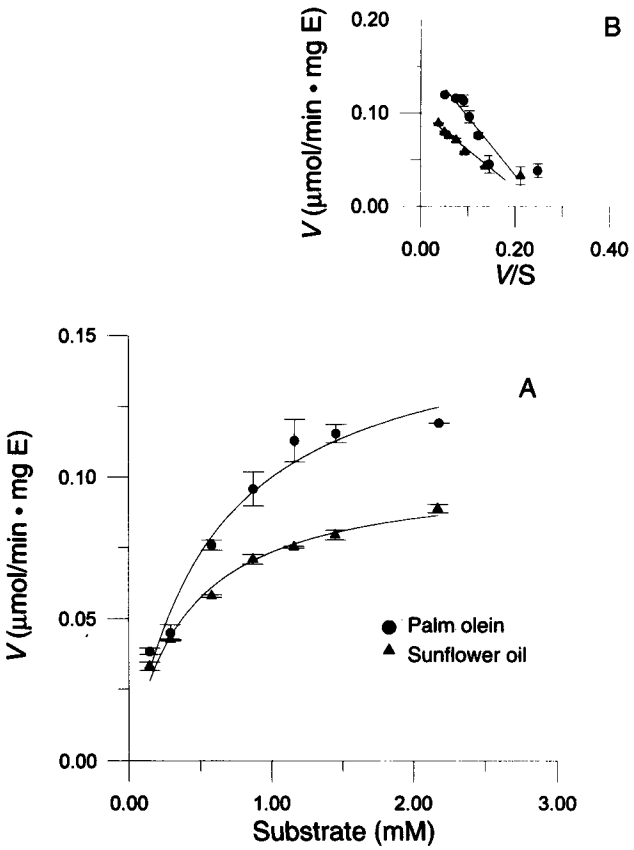


FIG. 2. (A) Activity of pancreatic porcine lipase toward palm olein (●) and sunflower oil (▲) both with approximately 18% of polar compounds (after sixty and thirty fryings, respectively). Conditions were: substrate ranging from $1.5 \cdot 10^{-1}$ mM to 2.2 mM, Enzyme = 1 mg/mL, 0.01 M Tris-HCl buffer (pH 8.3), T = 37°C. Solid line corresponds to computer fitting of the data to the Michaelis-Menten equation, and error bars refer to 95% confidence limits using the corresponding Student's t. (B) The same results under the Eadie-Hofstee plot.

Figure 2A shows that in both cases hydrolysis corresponds to a Michaelis-Menten saturation kinetic behavior, in agreement with the straight line observed in the Eadie-Hofstee plot (Fig. 2B). Each data set was fitted to the Michaelis-Menten equation by nonlinear regression and the corresponding V_{max}^{app} and K_M^{app} were estimated.

With a view to comparing the effect of the frying process of oils on the Michaelis-Menten parameters, Table 4 was constructed with the respective parameters.

As can be seen in Table 4 for palm olein, the K_M^{app} and V_{max}^{app} values did not show a clear relationship with the number of fryings, and therefore with the content of alterated compounds in the oils. Results of K_M^{app}/V_{max}^{app} suggest that within the range of our study, the enzymatic hydrolysis catalyzed by porcine pancreatic lipase in palm olein is not deeply modified by compounds of higher polarity (oxidized triacylglycerols) or high MW (triacylglycerol dimers and polymers). The new compounds yielded in oils during the culinary processes induced changes that did affect interphase quantity and quality.

Hydrolysis of alteration products by porcine pancreatic lipase has already been described by other authors. In this sense, Miyashita *et al.* (11) showed that porcine pancreatic lipase is not inactived in the presence of monohydroperoxides and that the hydrolysis preferentially takes place in monohydroperoxide fatty acids instead of nonoxidized fatty acids located in the same triacylglycerol position. Later, Henderson *et al.* (29) concluded that the high MW polymers of triacylglycerols present in oxidized fish oils can be hydrolyzed by pancreatic lipase *in vitro*. By contrast, authors such as Yoshida and Alexander (30) report that the lower the degree of enzymatic hydrolysis, the higher the complexity of the isolated fraction. Similarly, Márquez-Ruiz *et al.* (12) have demonstrated that the enzymatic hydrolysis of olive oil heated at 180°C depends on the MW of the glycerides, being slower as the MW of alteration compounds increases. Furthermore, it has been shown (31) that the polymers formed in fried oils have surfactant properties. Consequently emulsions with higher surfactant contents are more stable and their interphase surface is larger.

According to the above results, the alterations obtained after frying in palm olein induce different effects which may enhance or interfere with enzymatic hydrolysis. The possibility of which all these effects are balanced could be responsible for not clear and relevant changes on the kinetic parameters when studying various palm olein and sunflower oil samples presenting diverse alteration degrees, as it has been observed in this work (Table 4). Using sunflower oil as a substrate for porcine pancreatic lipase, although significant, no relevant changes in the kinetic parameters K_M^{app} and V_{max}^{app} of the catalytic process were induced by the increased alteration resulting from successive fryings (Table 4). These results are thus in agreement with these obtained for palm olein (Table 4).

On comparing the kinetic parameters obtained in the hydrolysis of palm olein and sunflower oil (Table 4), both K_M^{app}

TABLE 4
Kinetic Parameters for the Hydrolysis of Unused Palm Olein and Sunflower Oil and After Being Used in Successive Fryings of Potatoes Catalyzed by Pancreatic Porcine Lipase[a]

Oil	Number of fryings	K_M^{app} (mM) · 10	V_{max}^{app} (μmol/min · mg E) · 10^3	K_M^{app}/V_{max}^{app}
Palm olein	0	5.1 ± 0.7[a]	166 ± 7.6[a]	3.07 ± 0.56[a,b]
	60	5.9 ± 1.0[a]	159 ± 9.7[a]	3.71 ± 0.85[a]
	90	5.3 ± 0.9[a]	226 ± 14[b]	2.35 ± 0.54[b]
Sunflower	0	2.2 ± 0.3[a,b]	62.0 ± 2.2[a,b]	3.55 ± 0.61[a,b]
	30	3.9 ± 0.4[b,b]	102.0 ± 3.0[b,b]	3.82 ± 0.50[a]
	60	2.1 ± 0.4[a,b]	76.1 ± 3.2[c,b]	2.76 ± 0.64[b]

[a]Kinetic values for the same oil bearing different letter are significantly different.
[b]Significant respect to palm olein from the same number of fryings.

and V_{max}^{app} were higher in palm olein than in sunflower oil, regardless of the degree of alteration. The lower K_M^{app} value obtained with sunflower oil points to the higher enzymatic affinity of pancreatic lipase for this substrate as compared to palm olein, although the higher V_{max}^{app} value with palm olein showed a higher efficiency in hydrolyzing this oil. These differences are due to the different fatty acid composition in both culinary fats since all assays were performed under identical experimental conditions. Table 3 shows the fatty acid composition of both oils. Palm olein has a higher content of palmitic acid ($C_{16:0}$) and oleic acid ($C_{18:1}$) but a lower content of linoleic acid ($C_{18:2}$) as compared to sunflower oil. The differences in hydrolysis could be due to the oleic acid content since several authors have concluded that oleic acid is released with the highest relative speed (32).

To summarize, in the deep-fat frying of potatoes, thermooxidative reactions preferentially take place over hydrolytic reactions and consequently the degree of polymerization of culinary fats is increased. This originates an increase in the average MW of these oils. The methodology proposed by us is appropriated for calculating the MW of heat-oxidized oils. The alteration products arising during the frying process would not greatly affect the enzymatic hydrolysis, given by the apparent K_M and V_{max} values, of the triglycerides in oils.

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Chronic Free Fatty Acid Infusion in Rats Results in Insulin Resistance But No Alteration in Insulin-Responsive Glucose Transporter Levels in Skeletal Muscle

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ABSTRACT: To investigate the mechanism by which free fatty acids (FFA) affect glucose uptake, we studied the effect of chronic elevation (24 h) of plasma FFA in rats on whole body glucose disposal and glucose utilization index (GUI) in the basal state and under a euglycemic hyperinsulinemic clamp in relation to the amount of insulin-responsive glucose transporter (IRGT, i.e., GLUTU) protein in different muscles (oxidative and glycolytic) and adipose tissue. Infusion of Intralipid in the basal state led to a ~40% increase in whole body glucose uptake and a ~250% increase in GUI in adipose tissue as compared to control rats. There was no change in the amount of IRGT protein in any of the muscle types whereas in fat depots it was either unchanged or decreased. Under moderate or supraphysiological hyperinsulinemia, increment of whole body glucose disposal was significantly lower in Intralipid perfused rats when compared to controls (~110 μU/mL: 0.7 ± 0.1 vs. 1.3 ± 0.1 mg/min, $P < 0.02$; ~1000 μU/mL: 3.0 ± 0.2 vs. 3.9 ± 0.4 mg/min, $P < 0.02$). Under moderate hyperinsulinemia stimulation, GUI was significantly reduced in different muscles and adipose tissue as compared to controls. We conclude that peripheral insulin resistance which occurs after elevation of plasma FFA levels does not seem to be explained by changes in the amount of IRGT protein in either oxidative or glycolytic skeletal muscle. Thus fatty acid infusion appears to be associated with a defect in IRGT translocation to the plasma membrane, fusion with the membrane, or intrinsic activity.
Lipids 31, 1141–1149 (1996).

Over 25 years ago, Randle and his associates (1) demonstrated an inhibitory effect of free fatty acids (FFA) on glucose uptake in the perfused rat heart. Recently several groups have examined glucose-fatty acid interactions in humans (2–7) and rabbits (8) during hyperinsulinemia when most of the glucose uptake occurs in muscle (8). Some found inhibitory effects of lipid on whole body glucose uptake (5,6,9) but others did not (2–4,7). It was also observed that high FFA concentrations can even stimulate glucose uptake in the presence of hyperinsulinemia and euglycemia in rat skeletal muscle (10). Discrepancies between these studies could partly be explained by methodological differences. For example it was found that, in humans, the metabolic competition between FFA and glucose is very much time-dependent (4).

Assuming that FFA could interfere with glucose utilization, several investigators have attempted to explain the cellular mechanism by which FFA inhibit glucose uptake in skeletal muscle *in vivo* and *in vitro*. In humans, it was shown that lipid infusion reduced glucose uptake by interfering with glycogen synthesis, but it failed to increase citrate and glucose-6-phosphate concentrations (11). This latter observation does not support the Randle hypothesis. When the effect of FFA had been studied *in vitro*, it was shown that palmitate inhibited insulin-stimulated glucose transport only in fibers with a high respiratory capacity (9). It was suggested that palmitate could have a direct effect on the plasma membrane and could exert its inhibitory effects *via* alteration of the activity of the insulin-sensitive glucose transporter (IRGT, i.e., glucose transporter 4). Thus the interaction between FFA and glucose appears complex and seems to depend on the experimental conditions.

As previous experiments failed to demonstrate an inhibitory effect of fatty acids on insulin-mediated glucose uptake over a short period of time (2–4 h), we investigated whether a prolonged elevation (24 h) of FFA levels, which is often encountered in physiological or physiopathological situations, could solve the controversy over the issue of FFA/glucose interaction in rats. Furthermore we attempted to clarify which step of glucose uptake could be affected during hyperlipidemia by studying possible changes in expression of IRGT protein in oxidative and glycolytic muscle fibers.

MATERIALS AND METHODS

Animals. Three-month-old female Wistar rats weighing 200–220 g were used. They had free access to water and laboratory chow pellets (UAR 113, Villemoisson, France) containing 7% palmitic acid, 0.5% palmitoleic acid, 3% stearic

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Abbreviations: FFA, free fatty acids; GUI, glucose utilization index; IRGT, insulin-sensitive glucose transporter; Ra, rate of glucose appearance; Rd, rate of glucose disappearance.

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acid, 15% oleic acid, and 20% linoleic acid (calorific value 5 Kcal/kg). The technique for long-term infusion in unrestrained rats was used as described previously (12). The infusion period started on day 3 after surgery and lasted 24 h. Circulating concentrations of FFA were initially raised by a priming bolus of heparin (100 U) followed by a constant infusion of a mixture of a 10% triglyceride emulsion (Intralipid KabiVitrum, Helsingborg, Sweden; 2000 Kcal/L; 10% soybean oil containing 10% palmitic acid, 4% stearic acid, 23% oleic acid, 53% linoleic acid, 8% linolenic acid, 0.60 g lecithin, and 1.25 g glycerol per 100 mL and heparin 20 U/mL) at a rate of 50 μL/min/kg (=Intralipid rats). Control rats were infused with a saline solution. Basal state was defined as the end of saline or Intralipid infusion period (Fig. 1).

Glucose turnover rate. Measurements were performed at time 24 h while maintaining saline and Intralipid infusion (i.e., in the basal state) and during a euglycemic hyperinsulinemic clamp (protocol 1; Fig. 1). Rats were deprived of food for 4 h, i.e., under conditions of negligible glucose delivery by the gut (13). Rats were anesthetized with pentobarbital sodium (50 mg/kg i.p.). A catheter was inserted into the left carotid artery for blood sampling. Infusions (insulin, labeled and unlabeled glucose) were carried out using butterfly needles that were inserted in the saphenous veins.

Experiments consisted of a tracer ([3-^{3}H] glucose) equilibration period (−50 to −15 min), a basal period (−15 min to 0 min), and a euglycemic clamp study period (0 to 90 min). A priming dose of [3-^{3}H] glucose (4 μCi) was injected through the saphenous vein at −50 min followed by a continuous infusion at a rate of 0.2 μCi/min throughout the study. In Intralipid rats, Intralipid infusion was maintained throughout the study period (−50 to 90 min). Blood samples were drawn at −15, −10, and 0 min during the basal period and at time 70, 80, and 90 min during the steady-state euglycemic clamp for glucose disposal rate evaluation.

Euglycemic clamp. Before insulin infusion, two sets of blood samples were drawn for determinations of basal blood glucose, plasma insulin, and plasma FFA levels. A priming dose of insulin (20 mU; pork monocomponent insulin; Novo, Copenhagen, Denmark) dissolved in isotonic saline was injected through a saphenous vein followed by a continuous infusion of insulin (either 0.4 or 3 U/kg/h) at a constant rate of 20 μL/min.

During the glucose clamp, blood was sampled every 5 min to determine arterial glycemia and to adjust the rate of unlabeled glucose infusion to maintain the blood glucose concentration at the level measured in the basal state (i.e., 24 h after saline or Intralipid infusion). The euglycemic clamp was attained within 30–40 min and maintained for 60 min thereafter. Steady-state specific glucose radioactivity and plasma glucose and insulin concentrations were determined during the last 20 min of the clamp. Two additional blood samples were drawn for FFA determinations.

At completion of the euglycemic insulin clamp, animals were killed after i.p. administration of pentobarbital (150 mg/kg). The soleus (oxidative), gastrocnemius (glycolytic and oxidative separately), and tibialis anterior (glycolytic) muscles as well as white adipose tissue (periovarian and inguinal) and brown adipose tissue (interscapular site) were obtained for quantification of glucose transporter, IRGT. These muscles and the liver were also used for glycogen content determinations. All these parameters were also studied in the basal state (animals were killed prior to starting the euglycemic clamp). All tissues were immediately frozen at −80°C.

Glucose utilization index (GUI). GUI is defined as the amount of 2-deoxy glucose uptake by an individual tissue per unit of time. To determine the GUI, a tracer dose of 2-deoxy [1-^{3}H] glucose (25 Ci/mmol; 25 μCi/rat; CEA, Saclay, France) was injected in 100 μL of 0.9% NaCl as a bolus through a saphenous vein in animals submitted or not to an Intralipid infusion during 24 h (protocol 2; Fig. 1). At 1, 3, 5, 10, 20, 40, and 60 min after the injection, blood was sampled *via* the arterial catheter for determination of 2-deoxy [1-^{3}H] glucose radioactivity and blood glucose concentrations. Rats were killed by cervical dislocation 60 min after the 2-deoxy [1-^{3}H] glucose injection, and tissues were immediately removed and their 2-deoxy [1-^{3}H] glucose-6-phosphate contents were determined as described previously (14). Glucose utilization was then calculated, except that the value was not corrected by the discrimination factor (lumped constant) for 2-deoxyglucose in glucose metabolic pathways. Therefore the results are expressed as an index of glucose utilization.

Analytical methods. Blood glucose was determined by the glucose oxidase technique using a glucose analyzer (YSI, Yellow Springs, OH). For the assay of [3-^{3}H] glucose radioactivity, blood samples were deproteinized with $Ba(OH)_2$ and $ZnSO_4$, and the supernatant was evaporated to dryness at 50°C to remove tritiated water. The dry residue was dissolved in 0.5 mL water to which 10 mL scintillation solution were added (Aqualuma plus; Lumac, Arnhem, The Netherlands), and radioactivity was determined in a Packard Tri-Carb 460C liquid scintillation system. Plasma insulin concentrations were determined by a radioimmunoassay kit (SB-INSI-ORIS, Gif-sur-Yvette, France). FFA concentrations were measured using an enzymatic assay (NEFA-C test; Wako Chemicals, Neuss, Germany). Protein was assayed by Lowry's method

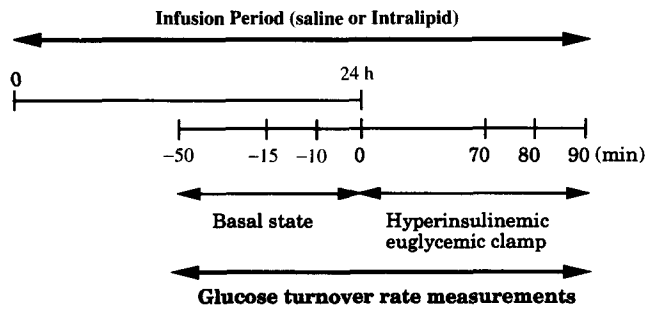


FIG. 1. Design of studies of measurements of glucose turnover rate (protocol 1) and glucose utilization index (protocol 2) in basal state and during the euglycemic/hyperinsulinemic clamp in rats.

(15), and glycogen concentrations in the liver and muscles were determined according to the technique of Roehrig and Allred (16). Muscle glycogen determinations required a previous digestion of the tissue (~50 mg) in NaOH (1 N) at 60°C for 1 h.

Homogenates from hindlimb muscles and adipose tissue. Each muscle was first homogenized with a Polytron (Janke & Kunkel, Staufen, Germany) in ice-cold phosphate-buffered saline (PBS; 0.9% NaCl, 20 mM KCl, 0.1 M phosphate buffer, pH 7.4) containing 1% Triton X-100 (Warren, MI). Homogenization was then completed with a glass homogenizer (Kontes, Hayward, CA) by 30 strokes. White and brown adipose tissue were homogenized in ice-cold 1 mM EDTA, 20 mM Tris/HCl, and 0.25 mM sucrose (pH 7.4).

Quantification of glucose transporter protein by immunoblotting. Equal amounts of proteins (100 µg) were solubilized in Laemmli buffer and subjected to a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were then electrophoretically transferred to nitrocellulose membranes (BA 85; Schleicher and Schuell, Dassel, Germany). Protein markers (Rainbow; Amersham, Bucks, United Kingdom) were used as molecular weight standards and also to assess the efficiency of the transfer. The membranes were blocked with nonfat dry milk (Carnation, Los Angeles, CA) in Tris (hydroxymethyl) aminomethane (Tris)-buffered saline (pH 7.6) with 0.1% Tween 20 tris buffer saline Tween 20 (TBST) and incubated at the recommended dilution of antibodies in the same solution washed three times in TBST, and reacted with ^{125}I-anti-mouse IgG (Amersham, Bucks, United Kingdom) for 1 h. The filters were again washed three times with TBST and exposed to Hyperfilm MP (Amersham) then quantified by scanning densitometry (connected to a Macintosh microcomputer; Hoeffer, San Francisco, CA). The antibody used was 1F8 (East-Acres Biological, Wilmington, MA) which is specific for the IRGT-protein.

Calculations. Glucose kinetics. In the basal steady state, the rate of glucose appearance (Ra, endogenous glucose production) was equal to the glucose disposal rate (Rd, glucose utilization). Rd was calculated according to the formula Rd = Ra = [3-^{3} H] glucose infusion rate [disintegrations per minute (dpm/min)] divided by blood glucose specific activity (dpm/mg), during the last 20 min of the glucose clamp (50–70 min after the onset of insulin infusion).

Results are given as means ± SE. Statistical analyses were performed with the unpaired Student's *t*-test.

RESULTS

During the lipid infusion, the mean food intake was significantly decreased compared with controls (11.8 ± 1.2 vs. 18.3 ± 2.3 g/day $P < 0.001$). In the Intralipid group, the daily energy intake was about 52.2 Kcal (14.4 and 37.8 Kcal from lipid and chow pellets, respectively). In control rats it was about 57.6 kcal. Total energy intake did not differ significantly between groups. The daily lipid intake was about 0.8 and 2.0 g in control and Intralipid rats, respectively.

It should be emphasized that a third group of rats was studied in postabsorptive state without previous saline infusion, i.e., 4 h after the food was withheld. All the parameters studied were not statistically different from those measured in the control group, thereby showing the absence of effect of saline infusion (data not shown).

Glucose, insulin, and FFA levels. In the basal state, i.e., 24 h after saline or lipid infusion, arterial blood glucose concentrations were 100 ± 1.6 and 102 ± 1.7 mg/dL, and insulin levels were 23.6 ± 1.6 and 24.7 ± 2.6 µU/mL in control and Intralipid rats, respectively. During the glucose clamp study, plasma insulin concentrations were raised to 107.1 ± 8.3 and 139.5 ± 15.0 µU/mL [nonsignificant (NS)] with a rate of 0.4 U/kg/h and to 1105 ± 115 and 1018 ± 124 µU/mL (NS) with a rate of 3 U/kg/h in control and Intralipid rats, respectively.

In the basal state, the arterial concentration of FFA was significantly higher in Intralipid than in control rats (Fig. 2). During hyperinsulinemia (0.4 U/kg/h), FFA levels in Intralipid rats remained similar to that measured in the basal state (0.76 ± 0.08 mM) whereas they were significantly lower (0.36 ± 0.03 mM $P < 0.001$) when infusing 3U/kg/h. In control rats, FFA concentrations were significantly decreased at both infusion rates of insulin (0.4 U: 0.23 ± 0.04 mM $P < 0.05$ and 3 U: 0.12 ± 0.02 mM $P < 0.001$) compared with basal state.

Glucose disposal rate. In the basal state, the glucose disposal rate was significantly higher in Intralipid than in control rats (Fig. 3A). During moderate hyperinsulinemia (~100 µU/mL), increment in glucose utilization was significantly lower in Intralipid than in controls (Fig. 3B). Under marked hyperinsulinemia (~1000 µU/mL), increment in glucose utilization averaged 3.0 ± 0.2 and 3.9 ± 0.4 mg/min ($P < 0.02$; Fig. 3B) in Intralipid and control groups, respectively.

Figure 4 depicts changes in glucose infusion rates during the glucose clamp. Under moderate (Fig. 4A) or marked (Fig. 4B) hyperinsulinemia, and over the time interval 30–90 min, the rate of glucose infusion was significantly lower in Intralipid rats than in controls. At completion of the two glucose clamp studies, the hepatic glucose production was totally suppressed in controls whereas it was decreased by ~50% in Intralipid rats (data not shown).

GUI. In a first group of rats, we measured glucose transport in individual tissues in order to know which ones had decreased glucose utilization after Intralipid infusion. In the basal state, i.e., after completion of saline and Intralipid infusions, the GUI of oxidative muscles (soleus and oxidative gastrocnemius) as well as glycolytic muscles (tibialis anterior and glycolytic gastrocnemius) were similar both in control and Intralipid rats (Fig. 5). In contrast, the GUI in white adipose tissue was more than two times higher ($P < 0.001$) in Intralipid than in control rats both in subcutaneous (inguinal fat pad) and profound (periovarian fat pad) locations. Note that GUI in brown adipose tissue was about five times higher than that measured in white adipose tissue, and it was unaffected by Intralipid infusion.

In a second group of rats, a euglycemic clamp was performed after 24 h while maintaining the Intralipid infusion.

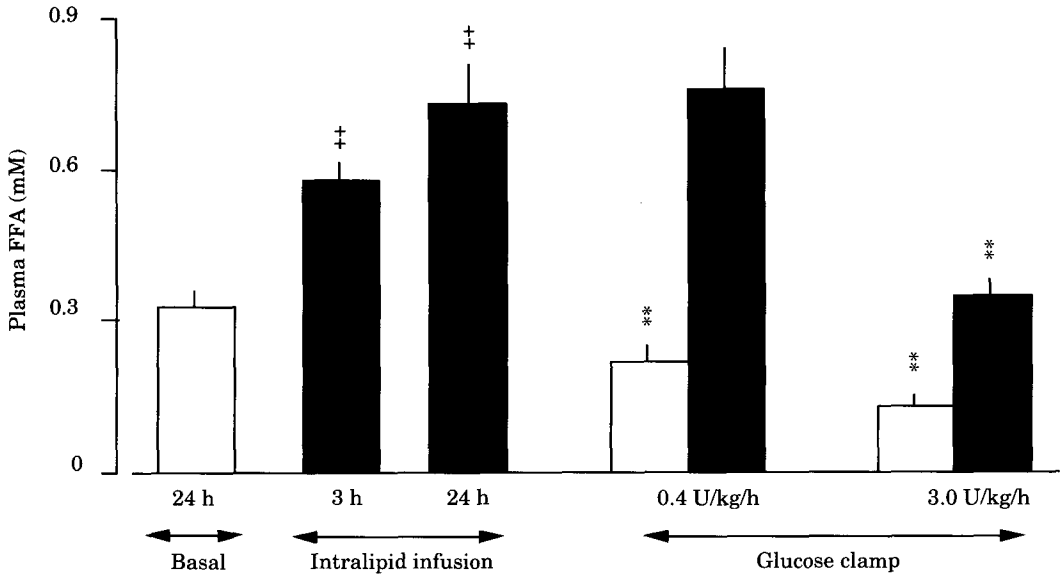


FIG. 2. Plasma free fatty acid concentrations in rats in the basal state, after saline (□, $n = 15$) or 24 h of Intralipid infusion (■, $n = 12$) and after a euglycemic hyperinsulinemic clamp performed at the end of infusion period. The rate of insulin infusion was either 0.4 or 3.0 U/kg/h, corresponding to plasma insulin levels of ~100 and 1000 μU/mL, respectively. $^{++}P < 0.001$ (Intralipid vs. control in the basal state); $^{**}P < 0.001$ (glucose clamp vs. basal state).

In control rats there was a 2–5-fold stimulation of glucose utilization by insulin in all muscles and fat depots. In contrast, in Intralipid rats, GUI was not significantly increased by insulin either in muscles (oxidative and glycolytic) or adipose tissue when compared to the basal state, indicating that insulin resistance took place in these tissues. With respect to the brown adipose tissue, it displayed a marked stimulation of glucose utilization in both groups, but it was less pronounced in Intralipid rats than in controls (46 ± 8 vs. 176 ± 18 ng/min/mg tissue).

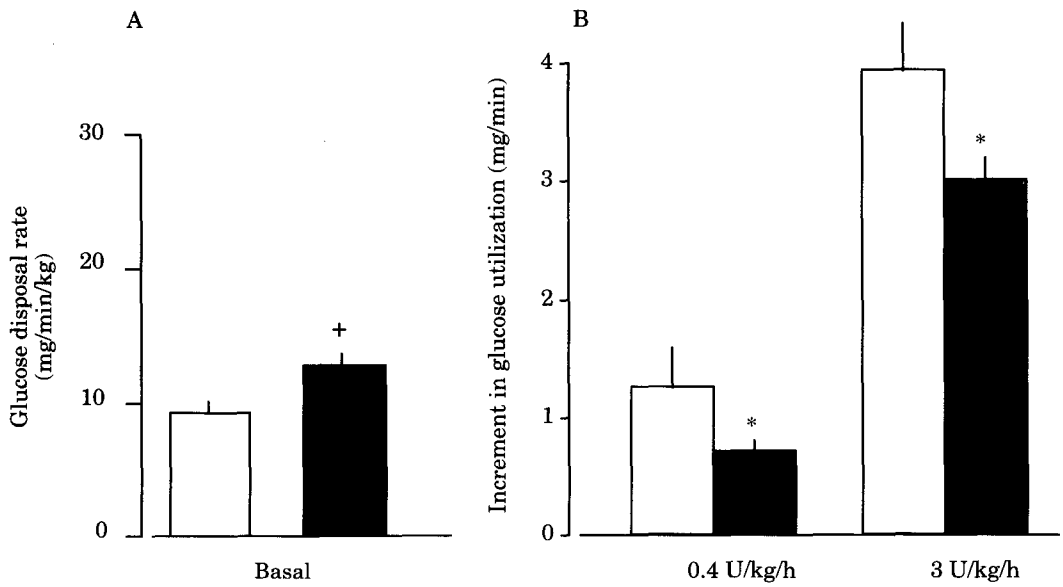


FIG. 3. Glucose disposal rate in rats, in the basal state (A) and increment in glucose utilization after a euglycemic hyperinsulinemic clamp (B). Saline (□, $n = 15$) or 24 h Intralipid infusion (■, $n = 12$). The rate of insulin infusion was either 0.4 or 3.0 U/kg/h, corresponding to plasma insulin levels of ~100 and 1000 μU/mL, respectively. A) $^{+}P < 0.01$ (Intralipid vs. control in the basal state); B) $^{*}P < 0.01$ (Intralipid vs. control after the glucose clamp).

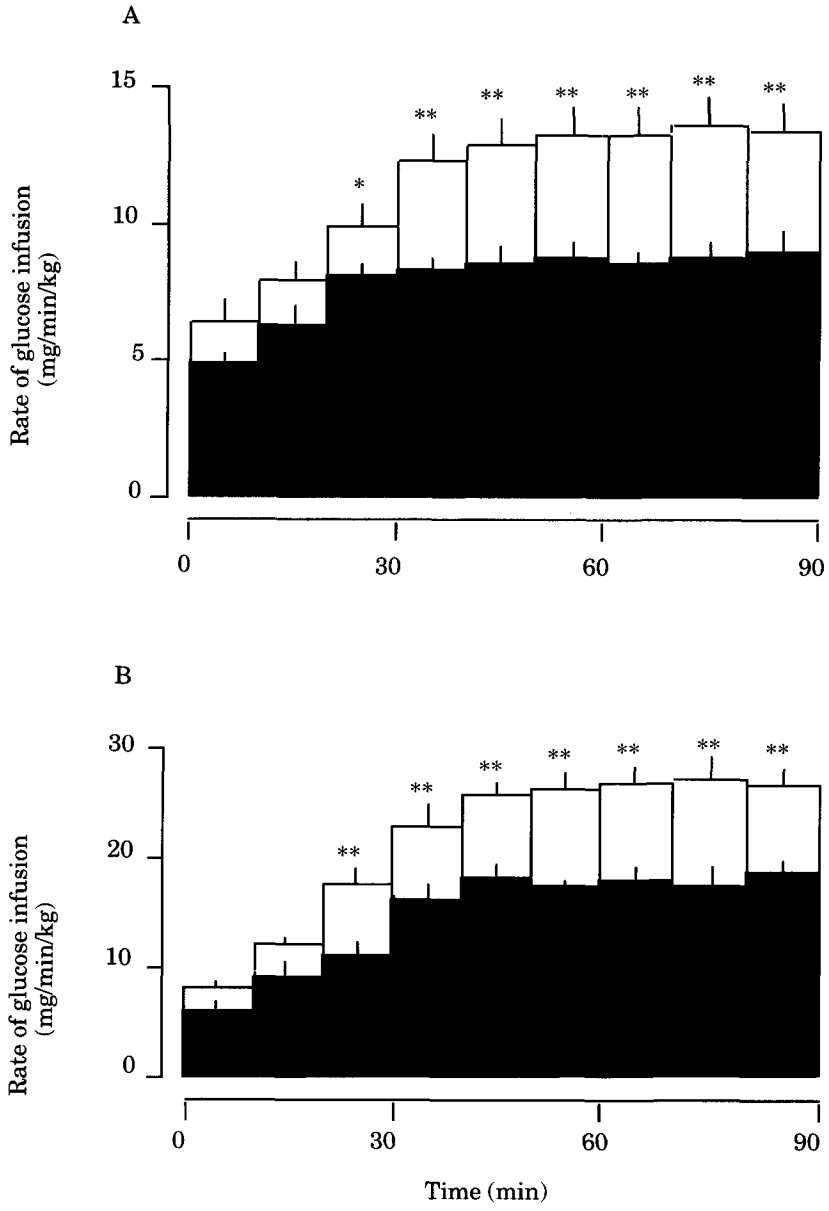


FIG. 4. Rates of glucose infusion during a euglycemic hyperinsulinemic clamp, in control rats (□, $n = 15$) and 24 h after Intralipid infusion (■, $n = 12$). The rate of insulin infusion was either 0.4 U/kg/h (A) or 3.0 U/kg/h (B), corresponding to plasma insulin levels of ~100 and 1000 μU/mL, respectively. $^{*}P < 0.01$, $^{**}P < 0.001$ (control vs. Intralipid).

Muscle and hepatic glycogen content. Lipid infusion significantly lowered hepatic glycogen content (Table 1). In contrast, it did not affect muscle glycogen content, and it was of the same order of magnitude (~4.5–5.0 mg/g) both in glycolytic and oxidative muscles. However when rats were given 11 g of food (restricted) which represents the intake of Intralipid group, without lipid infusion, muscle glycogen content was decreased by about 30% both in glycolytic and oxidative muscles when compared to Intralipid group.

Glucose-transporter concentration. Quantification of IRGT protein by densitometry showed that lipid infusion (basal state) did not affect the amount of transporters in each skeletal muscle type (Fig. 6A and 6B) as well as after moderate or marked hyperinsulinemia (data not shown). It also should be pointed out that the expression of IRGT in oxidative muscles (soleus and oxidative gastrocnemius) was much greater than in glycolytic muscles (tibialis anterior and glycolytic gastrocnemius). The highest expression of IRGT being in the soleus. In white and brown adipose tissue, lipid infusion did not affect the amount of IRGT except in the perirovarian fat pad in which it was significantly decreased ($P <$ 0.001 vs. control, Fig. 6C).

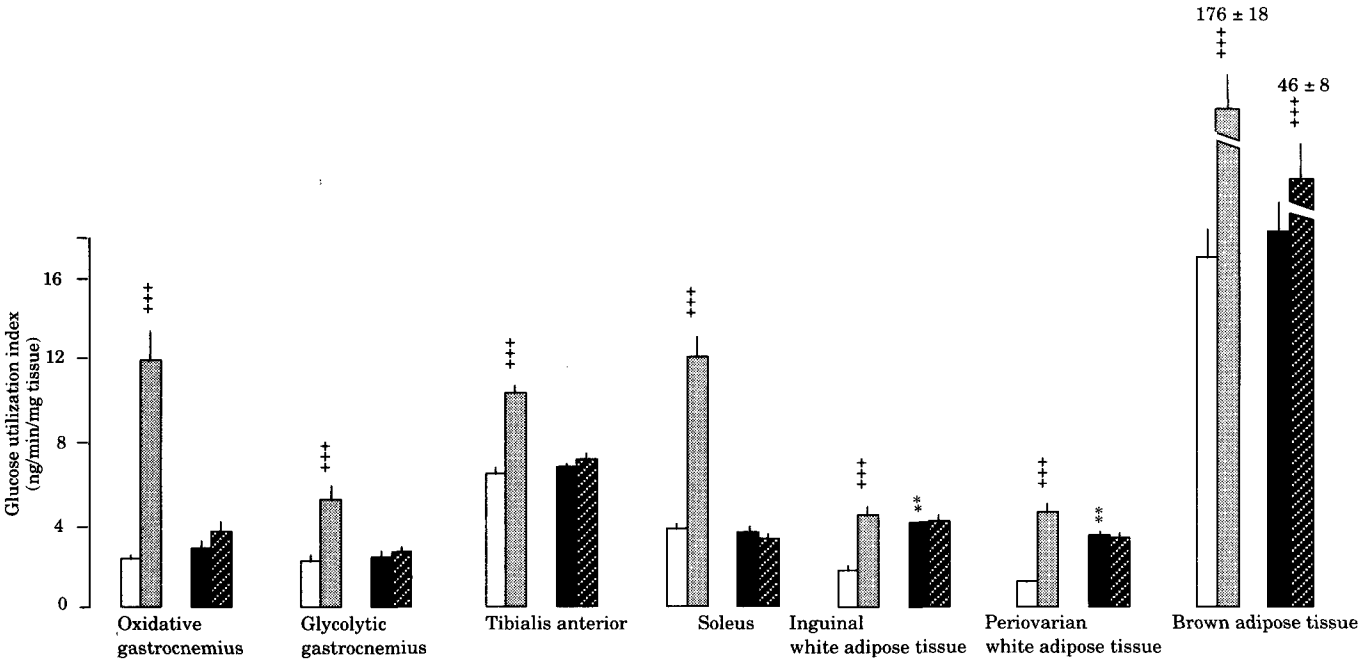


FIG. 5. Glucose utilization index in the basal state, i.e., at the end of saline or Intralipid infusion period (, control rats, $n = 8$; , Intralipid rats, $n = 7$) and during a glucose clamp performed after the end of saline or intralipid infusion period (, control rats, $n = 8$; , Intralipid rats, $n = 9$). $^{**}P < 0.001$ (control vs. Intralipid in the basal state); $^{+++}P < 0.001$ (glucose clamp vs. basal state).

DISCUSSION

The present work demonstrates that chronic elevation (24 h) of plasma FFA levels in rats results in an impaired insulin stimulation of GUI in skeletal muscles and adipose tissue. This defect cannot be attributed to changes in the amount of IRGT protein in skeletal muscles. However, since we measured total amount of IRGT protein (i.e., IRGT localized at the level of plasmic membrane and intracytosolic pool), we cannot rule out that FFA may interfere either with insulin by reducing the recruitment of functional IRGT or with IRGT function once translocated. It is also possible that glucose uptake was affected at a distal site. These unresolved issues need further investigation.

TABLE 1
Liver and Skeletal Muscle Glycogen Content (mg/g of tissue)[a]

	Control	Intralipid	Restricted
Muscles			
Oxidative gastrocnemius	5.01 ± 0.20	4.48 ± 0.30	3.50 ± 0.20*
Glycolytic gastrocnemius	4.77 ± 0.36	4.62 ± 0.30	3.40 ± 0.15*
Tibialis anterior	4.33 ± 0.25	4.76 ± 0.28	3.45 ± 0.20*
Soleus	4.90 ± 0.07	4.67 ± 0.30	3.41 ± 0.10*
Liver	56.30 ± 5.10	4.82 ± 1.13**	—

[a]Animals were perfused with saline (control), triglycerides (Intralipid) or were given 11 g of food which represent the intake of Intralipid group, without lipid infusion (restricted). Liver glycogen content was not measured in restricted rats.
*$P < 0.01$ food-restricted vs. Intralipid.
**$P < 0.01$ Intralipid vs. control.

In vivo and in the postabsorptive state (basal glucose and insulin levels), it seems that interactions between lipid and glucose metabolism operate under a restricted range of experimental conditions since it would be dependent upon FFA concentrations. Thus, in rats, inhibition of muscle glucose uptake occurs in the presence of physiological (moderate) and acute elevation in FFA levels (10). In contrast, it was found that supraphysiological concentrations of plasma FFA stimulate glucose utilization in rat skeletal muscle (10) and in human forearm tissues (17). In the present study, chronic elevation of FFA concentrations within a physiological range significantly increases whole body glucose disposal rate in the basal state.

During Intralipid infusion, enhanced glucose disposal rate occurs without change in blood glucose level. It implies an increase in hepatic glucose production, and it is mainly attributable to the increased gluconeogenesis since the liver is almost depleted in glycogen stores. *In vivo* studies have shown that the rates of FFA/lipid oxidation are positively correlated with the rate of hepatic glucose production (18). *In vitro* studies also demonstrated that the addition of palmitate to hepatocytes stimulates gluconeogenesis (19) and partially inhibits glycolysis (20). Furthermore, increased fat oxidation provides the stimulus for the activation of the key enzymes of gluconeogenesis (21). Taken together these factors may account for most of the increased hepatic glucose production.

In the basal state, FFA increased rather than decreased glucose disposal rate. This is inconsistent with the operation of the glucose–fatty acid cycle. Using the 2-deoxyglucose technique, we showed that the uptake of glucose by different mus-

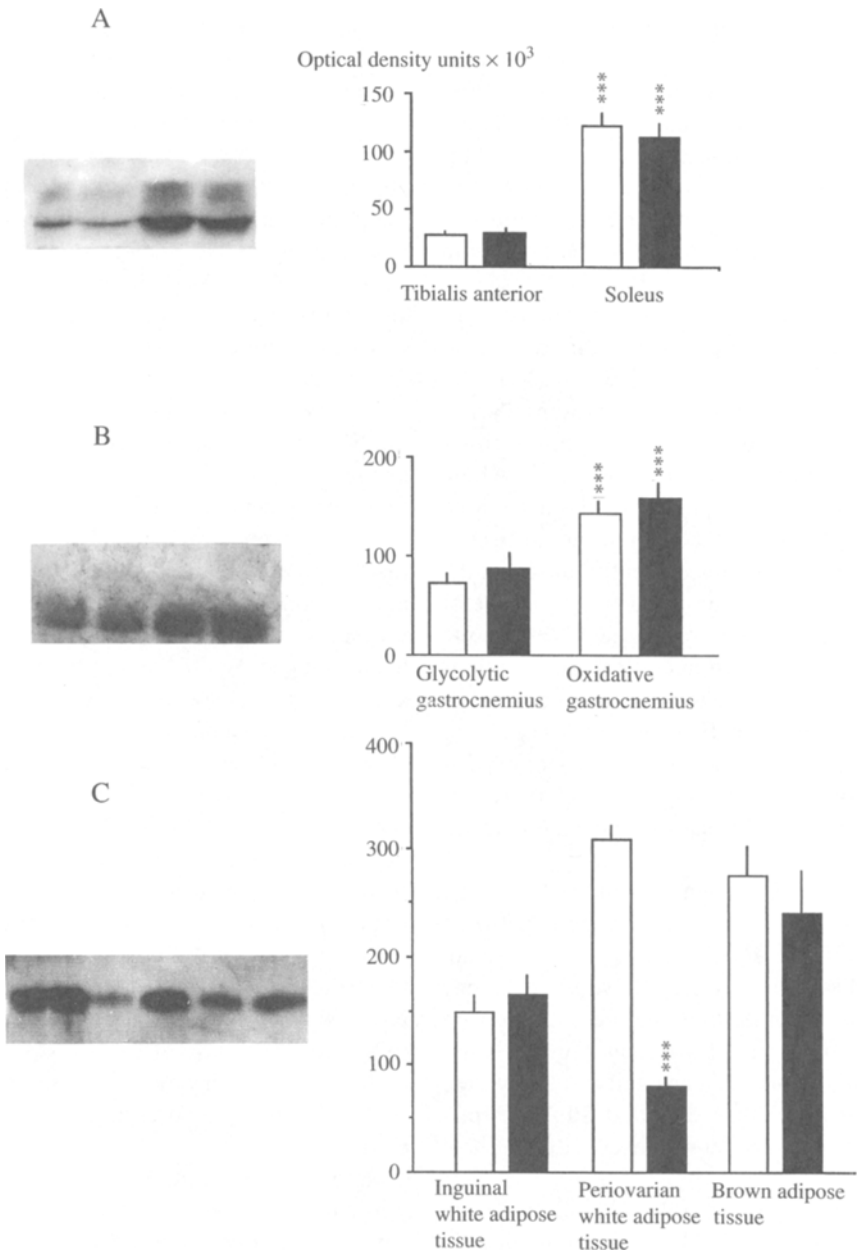


FIG. 6. Effect of Intralipid infusion (basal state) on the amount of IRGT protein in different muscles of the hindlimb and adipose tissue in control (□, $n = 5$) and in Intralipid (■, $n = 5$) rats in the basal state. IRGT protein was determined using a Western blotting protocol and detection of IRGT using a specific anti-serum. (A): ***$P < 0.001$ (soleus vs. tibialis anterior) (B): ***$P < 0.001$ (oxidative gastrocnemius vs. glycolytic gastrocnemius). (C): ***$P < 0.001$ (control vs. Intralipid rats).

cle types was unchanged. This observation implies that other tissues should augment their glucose utilization. Adipose tissue would be a potential candidate because FFA at physiological concentrations have been reported to increase basal glucose transport in isolated rat adipocytes (9,22,23) and *in vivo* when acutely increasing the circulating FFA concentrations in rats (10). In the present study we also found an increased glucose uptake in white adipose tissue (subcutaneous and profound) in Intralipid group as compared to control rats. From these previous studies (9,10), a greater uptake of glucose for reesterification in the face of increased FFA influx would seem the logical explanation for the increase in glucose disposal rate. However a rough estimate of the increment in GUI in white adipose tissue (Fig. 5) indicates that it cannot account for the totality of the increase in glucose disposal rate (Fig. 3). Consequently, it seems logical to suggest that some non-insulin-sensitive tissues also increased their glucose uptake.

Under euglycemic hyperinsulinemic conditions, we con-

firmed that the stimulation of whole body glucose uptake is markedly impaired in the presence of elevated plasma FFA concentrations (3,6,8). These data could be of clinical relevance, particularly in view of the recent demonstration that elevation of blood FFA concentration produced peripheral insulin resistance in healthy volunteers (24) and in noninsulin-dependent diabetic patients (25). Using the 2-deoxyglucose technique, we emphasize that insulin resistance takes place in all insulin-sensitive tissues studied, i.e., both muscles (glycolytic and oxidative) and adipose tissue (white and brown). Similarly, studies carried out on isolated rat adipocytes also demonstrated that prolonged treatment with saturated fatty acids induced insulin resistance (22). In the present work it was unexpected to note that under moderate hyperinsulinemia there was a modest but significant increment in whole body glucose disposal rate whereas there was no detectable stimulation of 2 deoxyglucose uptake in individual tissues studied. Such a discrepancy remains currently unexplained. We can only hypothesize that the discrimination factor (lumped constant) for 2-deoxyglucose in glucose metabolic pathways is lower in Intralipid than in control rats. Previous studies showed that in VMH obese rats the lumped value in white adipose tissue was significantly lower in VMH as compared to control rats (26). If it applies to the present experiment it would mean that insulin stimulation of the 2-deoxyglucose uptake by individual tissues is underestimated in the Intralipid group.

With respect to the muscle glycogen content, we found that elevated FFA level seems to have a "glycogen sparing effect." Thus, in another group of food restricted rats which were not infused with Intralipid but which were allowed to consume 11 g of food as in the Intralipid group, the muscle glycogen content declined by ~30%. Although we cannot precisely determine the mechanism(s) which can account for this finding one can however postulate that elevated FFA inhibits glycogenolysis as has been shown during exercise in skeletal muscle (27).

In parallel with the changes in whole body glucose disposal we studied the amount of IRGT protein in glycolytic and oxidative muscles in the basal state (i.e. after saline and lipid infusion). After saline infusion, expression of IRGT is much greater in oxidative muscles (soleus and oxidative gastrocnemius) than in glycolytic muscles (tibialis anterior and glycolytic gastrocnemius) which is in keeping with previous work (28). When lipid was infused both the expression of IRGT and the GUI did not change in each type of muscle when compared to controls in spite of an increase in whole body glucose uptake. This is in keeping with a recent study that showed that palmitate does not affect glucose transport in either the soleus or epitrochlearis *in vitro* in the absence of insulin (9). Consequently, other tissue(s) could significantly enhance their glucose uptake. White adipose tissue would be involved since its 2-deoxyglucose uptake is increased after Intralipid infusion. However, it is not accompanied by parallel changes in IRGT expression because the amount of transporter is either unchanged (inguinal adipose tissue) or decreased (periovarian tissue). It has been demonstrated that increased glucose transport does not necessarily imply changes in the amount of IRGT in the plasma membranes. Thus, in isolated rat adipocytes, both palmitate and oleate stimulate glucose transport but only palmitate increases the amount of IRGT in the plasma membranes (9,23). This finding points out that the stimulatory effect of oleate on glucose uptake in adipocytes may be mediated by changes in the intrinsic activity of IRGT. In the present work the same conclusion could be extended to the white adipose tissue. However it is also possible that the amount of glucose transporter 1 was increased.

The role of IRGT in the insulin resistance phenomenon has not been clearly established yet. In humans, when inducing an insulin-resistant state by infusion of lipid, there is no relationship between glucose uptake and the content of IRGT in skeletal muscle during 3 h of euglycemia and hyperinsulinemia as well as in the postabsorptive state (29). In animal models it seems that variations in the amount of IRGT protein depends on the way the insulin-resistant state has been induced as well as the type of muscle studied. Thus a four-day hyperinsulinemia imposed on normal rats in the presence of moderate hypoglycemia produced an insulin-resistant glucose transport in several muscles (30) as well as a decrease of the glucose transporter protein in the tibialis (glycolytic muscle) (31). When hyperinsulinemia and hyperglycemia coexist for three days in rats, there is no change in IRGT protein in hindlimb muscle, which suggests that impaired insulin action was probably due to decreased intrinsic activity of the transporter (32). Finally, the insulin-resistance glucose uptake characteristic of obesity and NIDDM cannot be attributed to altered expression of IRGT (33,34).

In summary, chronic elevation of plasma FFA levels results in (i) an impaired insulin stimulation of the GUI in muscle and adipose tissue and (ii) unchanged amount of IRGT protein in different muscle types (glycolytic and oxidative). These results are consistent with changes in IRGT functional activity and/or compartmentalization or a defect at the level of intracellular glucose metabolism.

ACKNOWLEDGMENTS

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Highly Hydrogenated Dietary Soybean Oil Modifies the Responses to Polychlorinated Biphenyls in Rats

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ABSTRACT: The effects of dietary highly hydrogenated soybean oil (HSO) upon the changes caused by dietary polychlorinated biphenyls (PCBs) were examined in rats. Six groups of rats were fed the following diets for 30 d: a 20% soybean oil-containing diet (control diet), a diet in which a half of soybean oil was substituted with HSO (HSO-A diet), a diet in which cellulose powder was replaced with HSO (HSO-B diet) and these diets supplemented with 100 ppm PCBs (control + PCBs, HSO-A + PCBs and HSO-B + PCBs diets). Hepatic concentration of PCBs and relative liver weight were markedly decreased in rats fed with the HSO-A + PCBs diet compared with those fed with the other diets containing PCBs. Liver lipids and liver cholesterol were considerably decreased with a reciprocal increase in fecal sterol excretion by rats fed the HSO-A + PCBs and the HSO-B + PCBs diets compared with those fed with the control + PCBs diet. The fatty acid composition in hepatic phospholipids showed an independent increase of the saturated fatty acid content induced by dietary HSO and PCBs. Dietary PCBs also caused decreases in the amounts of monounsaturated and n-3 polyunsaturated fatty acids. These results suggest that dietary HSO prevents accumulation of PCBs in the liver and promotes the excretion of lipids stimulated by PCBs, accompanied by a change in fatty acid metabolism.
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Polychlorinated biphenyls (PCBs) are worldwide environmental pollutants. Because of a variety of physicochemical properties depending upon the degree of chlorination, commercial PCBs have been used for a wide range of purposes, for example, as heat transfer fluids, dielectric fluids for transformers and capacitors, organic diluents, lubricants, etc. Although the total amount of PCBs in the world was estimated in the past at about 1.5 million tons (1), there is no information about the amount of PCBs which have leaked from various facilities or products into the environment. In Japan these compounds have not been produced for many years, but they still persist in the environment and food. PCBs in the environment have therefore been transported and accumulated into living organisms, which have experienced various undesirable effects. Recently nonortho-chlorine substituted (coplanar) PCBs have been recognized as toxic as polychlorinated dibenzo-*p*-dioxins (2), and these findings have caused a new awareness of the risk of PCBs.

Commercial PCBs (Aroclor, Kanechlor, or Clophen) have a broad spectrum of toxicity that depends upon the chlorine content of the mixture, animal species, and exposure duration to the mixture. The toxic effects of PCBs included acute lethality, hepatomegaly, fatty liver, porphyria, dermatological aberrations, immunotoxicity, carcinogenesis, and neurotoxicity. Hepatotoxicity, in relation to lipid metabolism, is the general response observed in diverse laboratory animal species including rats, mice, rabbits, guinea pigs, monkeys, and minks. For example, toxic responses including metabolic abnormality of lipid metabolism in liver were observed in rats (3) and a monkey (4) exposed to low levels of chronic PCBs. Besides these direct effects, dietary PCBs also affect several nutritional events. Horio *et al.* (5,6) have shown that ascorbic acid levels in the liver and plasma are reduced by feeding PCBs to rat mutants unable to synthesize ascorbic acid, whereas ascorbic acid formation in normal rats is markedly enhanced by dietary PCBs (7,8). Other antioxidants such as β-carotene (9) or tocopherols (10) are also decreased significantly by PCBs in laboratory animals. According to Kato and Yoshida (11), feeding PCBs to rats causes cholesterol accumulation in the plasma and liver with an increase in its biosynthesis. We have also reported the effects of dietary cholesterol, ubiquinone-10, or powdered green tea on the metabolic responses to dietary PCBs (12–14).

On the other hand, we found that highly hydrogenated soybean oil (HSO), considered to be hardly digested and poorly absorbed, also prevented cholesterol accumulation in the liver and promoted its rapid excretion in rats (15). These results indicate that HSO might also prevent the absorption of certain xenobiotics and reduce their adverse effects, at least in part.

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Abbreviations: HDL, high density lipoprotein; HSO, highly hydrogenated soybean oil; MUFA, monounsaturated fatty acids; PCBs, polychlorinated biphenyls; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

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In this paper, therefore, we studied how dietary HSO affected the altered lipid distribution, including cholesterol transport, caused by dietary PCBs.

MATERIALS AND METHODS

Animal experiments. Male Wistar rats (Japan SLC Inc., Hamamatsu, Japan), weighing an average of 80 g, were housed individually in cages with a wire screen bottom and were provided with a commercial nonpurified diet (CLEA Japan Inc., Tokyo, Japan) and tap water *ad libitum* before the experiment for 5 d. A room temperature was maintained at 22–24°C on a 12-h light/dark cycle with light from 1300–0100. At the end of the experiment, feces were collected for 48 h just before fasting. After fasting overnight, rats were anesthetized with diethyl ether and sacrificed between 0700–0800. Blood was collected from the abdominal aorta, using a plastic syringe containing heparin, for plasma cholesterol analysis. Immediately after the livers were removed, weighed and cooled on ice, they were frozen at –70°C until assay.

All aspects of the animal experiments were approved by the animal policy and welfare committee in our Institute.

The composition of experimental diets and that of fatty acids in dietary fats are shown in Tables 1 and 2, respectively. The diet in which 50% of soybean oil was replaced with highly HSO was named HSO-A and that in which cellulose powder was completely replaced by HSO was named HSO-B. To prepare diets containing PCBs, Kanechlor 500 (Kaneka Co. Ltd., Osaka, Japan) was added to each diet at a concentration of 100 ppm. The composition of PCB congeners in Kanechlor 500 was similar to Arochlor 1254 which contains 54% of chlorine (w/w).

Analysis. PCBs were determined by the method of Ukawa *et al.* (16) using a gas–liquid chromatograph with an electron capture detector (HP5890 series II; Hewlett-Packard Co., Palo Alto, CA) with SPD-1 (Sperco Inc., PA). Total cholesterol and high density lipoprotein (HDL)-cholesterol in plasma obtained from blood centrifuged at 1500 × *g* for 10 min were measured using commercially available kits (Cholesterol C-test and HDL-cholesterol C-test; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Liver lipid extracted by the method of Folch *et al.* (17) was determined gravimetrically. Fecal lipid was extracted by modified method of Folch *et al.* (17) in which the extraction process was performed using warm chloroform–methanol solution. Lipid in the freeze-dried feces was extracted by warm Folch solution containing a small amount of water (60°C, 1 h). After being dried under an N_2 stream, the residue was dissolved in 25 mL petroleum ether with anhydrous Na_2SO_4. Twenty mL of petroleum ether phase was transferred to a receptacle which was weighed in advance, and evaporated under a stream of N_2 followed by drying in an oven (100°C, 1 h). Lipid was determined by the difference between the initial and final weight of the receptacle. After mild saponification of the lipids, liver cholesterol was extracted and determined by the method of Grundy *et al.* (18). Fecal sterols were extracted by the method of Yamanaka *et al.* (19). Fecal neutral sterols were assayed by the procedure of Grundy *et al.* (18). The concentration of total fecal acidic steroids was assayed by the method of Forman *et al.* (20). After separation from other lipids with thin-layer chromatography (21), hepatic phospholipid was methylated with 5% HCl–methanol at 100°C for 3 h, and the fatty acid composition was determined by the procedure of Kawai and Nakayama (22) with the slight modification of using a slower temperature gradient program in capillary gas chromatography (4°C/min from 100 to 230°C). Urinary ascorbic acid was assayed by the method of Okamura (23). Hepatic cytochrome P-450 was determined by the procedure of Omura and Sato (24). Frozen liver slices were stained with Oil Red for the histochemical observation according to the procedure of Lillie (25).

TABLE 1
Composition (%) of Experimental Diets

	Diet		
	Control	HSO-A	HSO-B
Milk casein	20	20	20
α-Corn starch	25	25	25
Sucrose	20	20	20
AIN-Vitamin mix	0.9	0.9	0.9
AIN-Mineral mix	4	4	4
Choline-Cl	0.1	0.1	0.1
Soybean oil	20	10	20
Hydrogenated soybean oil (HSO)		10	10
Cellulose powder	10	10	

TABLE 2
Fatty Acid Composition of Dietary Fats

	Fatty acid composition (%) of experimental diets								
	16:0	18:0	18:1	18:2	18:3	20:0	22:0	SFA[a]	UFA[a]
Control	10.6	3.8	23.9	53.2	7.5	0.5	0.5	15.5	84.6
HSO-A	11.3	44.8	12.2	26.6	3.7	0.8	0.6	57.5	42.5
HSO-B	11.0	31.2	16.1	35.5	5.0	0.7	0.5	43.4	66.6

[a]SFA and UFA, saturated fatty acid and unsaturated fatty acid, respectively. See Table 1 for other abbreviation.

RESULTS

Body weight gain during the experiment (30 d) was similar among rats fed with any of the three diets regardless of the addition of PCBs (control: 141 ± 4.4 g, control + PCBs: 140 ± 5.8 g, HSO-A: 139 ± 6.1 g, HSO-A + PCBs: 140 ± 5.2 g, HSO-B: 139 ± 3.8 g, HSO-B + PCBs: 138 ± 2.4 g). Food consumption was about 20% greater in rats given the HSO-A diet (HSO-A: 501 ± 16.8 g, HSO-A + PCBs: 502 ± 16.0 g) than in those given the other two diets with or without PCBs (control: 413 ± 7.9 g, control + PCBs: 420 ± 8.6 g, HSO-B: 405 ± 6.7 g, HSO-B + PCBs: 413 ± 7.0 g). The effects of dietary HSO and PCBs on liver weight, lipids, and plasma cholesterol are shown in Table 3. Addition of PCBs to the diets increased the relative liver weight of rats, but the increase was least in the HSO-A + PCBs diet group among the three. The concentration of liver lipid was increased by the addition of

TABLE 3
Effects of Dietary PCBs and Highly Hydrogenated Soybean Oil on Relative Liver Weight, Plasma and Liver Lipids in Rats[a]

	Control	Control + PCBs	HSO-A	HSO-A + PCBs	HSO-B	HSO-B + PCBs
Relative liver wt (g/100 g b.w.)	3.9 ± 0.08[a]	5.9 ± 0.12[b]	3.9 ± 0.07[a]	5.0 ± 0.05[c]	3.9 ± 0.07[a]	5.8 ± 0.13[b]
Liver lipid (mg/liver/100 g b.w.)	448 ± 27.3[a]	636 ± 46.6[b]	359 ± 28.7[c]	425 ± 32.8[a]	422 ± 14.4[a]	528 ± 34.9[d]
Liver cholesterol (mg/liver/100 g b.w.)	7.9 ± 1.69[a]	53.2 ± 0.82[b]	6.5 ± 0.24[c]	22.2 ± 1.75[d]	9.3 ± 1.02[a]	24.5 ± 1.99[d]
Plasma total cholesterol (mg/dl)	50 ± 3.3[a]	72 ± 5.5[b]	54 ± 4.7[a]	72 ± 3.7[b]	47 ± 3.3[a]	78 ± 5.3[b]
Plasma HDL-cholesterol (mg/dl)	32 ± 2.0[a]	52 ± 3.4[b]	34 ± 2.5[a]	51 ± 2.6[b]	32 ± 1.2[a]	50 ± 2.5[b]

[a]Each value shows mean ± SEM, $n = 6$. In each experiment, values on the same line across without common superscript letters denote significant differences ($P < 0.05$). PCBs, polychlorinated biphenyls; HDL, high density lipoprotein. See Table 1 for other abbreviation.

PCBs to the diet, but this accumulation of liver lipid was prevented by the concomitant ingestion of HSO, especially by the HSO-A + PCBs diet. Moreover, under PCB-free conditions, the concentration of liver lipid was lower in rats fed with the HSO-A diet than in rats fed with the other two diets. A considerable increase in liver cholesterol level caused by supplementary PCBs was markedly prevented by HSO. Effects of dietary HSO and PCB on fecal weight and sterols are shown in Table 4. Rats fed HSO-A excreted more than twice the sterols of that by the other groups. As shown in Table 4, fecal total neutral sterols, cholesterol and coprostanol, were kept low and at similar levels among rats fed with the three diets without PCBs, but were increased considerably by supplementing with PCBs. Moreover, HSO ingestion changed the composition of neutral sterols in feces. Coprostanol was excreted at considerable levels into the feces of rats given the control diet with or without PCBs, whereas it was undetectable in the feces of animals given HSO regardless of PCBs supplement. Rats given the PCB-containing diets had higher total plasma cholesterol and HDL-cholesterol than those given the corresponding PCB-free diets (Table 2), with no significant difference among the groups given PCB diets. The amounts of urinary ascorbate and the level of hepatic cytochrome P-450 increased by dietary PCBs were not affected by HSO ingestion (ascorbic acid shown by mg/day/100 g body weight in control, control + PCBs, HSO-A, HSO-A + PCBs, HSO-B, HSO-B + PCB were 0.08 ± 0.003, 8.46 ± 0.794, 0.10 ± 0.014, 7.35 ± 0.702, 0.09 ± 0.010, and 8.01 ± 0.585, respectively, and cytochrome P-450 shown by nmol/mg protein in control, control + PCBs, HSO-A, HSO-A + PCBs, HSO-B, HSO-B + PCB were .0.5 ± 0.12, 2.0 ± 0.19, 0.5 ± 0.02, 1.8 ± 0.09, 0.7 ± 0.07, and 2.1 ± 0.13, respectively).

The effect of dietary HSO on PCB accumulation in rat liver are shown in Figure 1. Although a considerable amount of PCBs accumulated in the livers of rats given PCBs-containing diets, the PCB level was about half in rats on HSO-A + PCBs diet compared with those on the other two diets with PCBs. PCBs were undetectable in the livers of rats on the PCB-free diets.

Figure 2 shows the typical photomicrographs of liver sections stained with Oil Red from rats given the control and experimental diets. The panlobular region was evenly stained with Oil Red in rats given the control diet. However, in the presence of HSO, the staining was limited to only the midlobular region between the periportal and perivenous region in rats given especially the HSO-A diet. By the addition of PCBs to the diets, the staining profile was distinctly changed around the central vein. Similar, but less apparent changes were evident in rats given the HSO-A + PCBs diet.

Table 5 shows the fatty acid composition of hepatic phospholipids in rats given the control and the HSO-A diet with or without PCBs. HSO intake increased total saturated fatty acids, especially 18:0, though it decreased polyunsaturated fatty acids, mainly 18:2. Addition of PCBs to the diets also increased saturated fatty acids, especially 18:0, whereas it decreased 18:1 and n-3 polyunsaturated fatty acids with changes in the n-6 polyunsaturated fatty acids, namely an increase in the 20:4 n-6 and a decrease in the 18:2n-6. Consequently, dietary HSO and PCBs had additional effects on the 18:0 content of liver phospholipid. The ratio of n-6/n-3 was increased by dietary PCBs, but not by HSO.

DISCUSSION

There was no apparent difference in body weight gained, but food consumption was significantly greater in rats given the HSO-A diet than in those given the other two diets (Table 3). By analysis of the HSO used in our experiment, 57% of the triacylglycerol in HSO was tristearoylglycerol and most of

TABLE 4
Effects of Dietary PCBs and Highly Hydrogenated Soybean Oil on Fecal Dry Weight and Fecal Sterols in Rats[a]

	Control	Control + PCBs	HSO-A	HSO-A + PCBs	HSO-B	HSO-B + PCBs
Fecal dry weight (g/day/100 g b.w.)	0.59 ± 0.023[a]	0.62 ± 0.025[a,c]	1.33 ± 0.028[b]	1.36 ± 0.033[b]	0.54 ± 0.006[a]	0.66 ± 0.025[c]
Fecal cholesterol (mg/day/100 g b.w.)	0.5 ± 0.03[a]	4.8 ± 0.32[b]	2.1 ± 0.16[c]	5.1 ± 0.21[b]	2.5 ± 0.12[c]	7.4 ± 0.48[d]
Fecal coprostanol (mg/day/100 g b.w.)	1.3 ± 0.09[a]	1.0 ± 0.07[b]	n.d.	n.d.	n.d.	n.d.
Fecal total bile acids (mg/day/100 g b.w.)	0.7 ± 0.07[a]	2.7 ± 0.10[b]	0.9 ± 0.13[c]	3.1 ± 0.16[b]	1.1 ± 0.14[c]	2.4 ± 0.11[d]

[a]Each value shows mean ± SEM, $n = 6$. In each experiment, values on the same line across without common superscript letters denote significant differences ($P < 0.05$). n.d. = not detected. See Tables 1 and 3 for abbreviations.

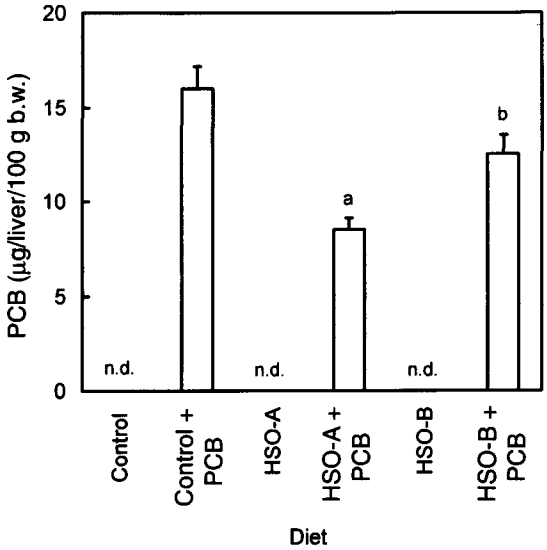


FIG. 1. Effect of dietary polychlorinated biphenyl (PCB) and highly hydrogenated soybean oil on PCB accumulation in the liver; n.d. = not detected. Superscript letters denote significant difference ($P < 0.05$).

the remainder distearoylmonoacylglycerols (15). As the HSO-A diet contained a greater amount of tristearoylglycerol which is considered to be difficult to digest (26), rats given the HSO-A diet would have to eat more food to obtain a sufficient growth rate and hence excreted more in the feces as shown in Table 5. Rats on the HSO-A + PCBs diet had the lowest relative liver weight and PCBs accumulation in the liver among rats on the three diets containing PCBs (Fig. 1). The indigestibility and low absorptivity of HSO which induces the suppression of the diffusion of PCBs through the intestinal wall and their rapid passage through the bowels appeared to prevent the absorption of dietary PCBs.

HSO did not prevent the increase in plasma total cholesterol caused by dietary PCBs. The effect of dietary HSO on the transport of endogenous cholesterol might differ from that of exogenous cholesterol. A different type of lipoprotein metabolism might be involved, but details were unclear from the present experiment.

The histochemical results showed that the shape and arrangement of hepatocytes in the perivenous region were al-

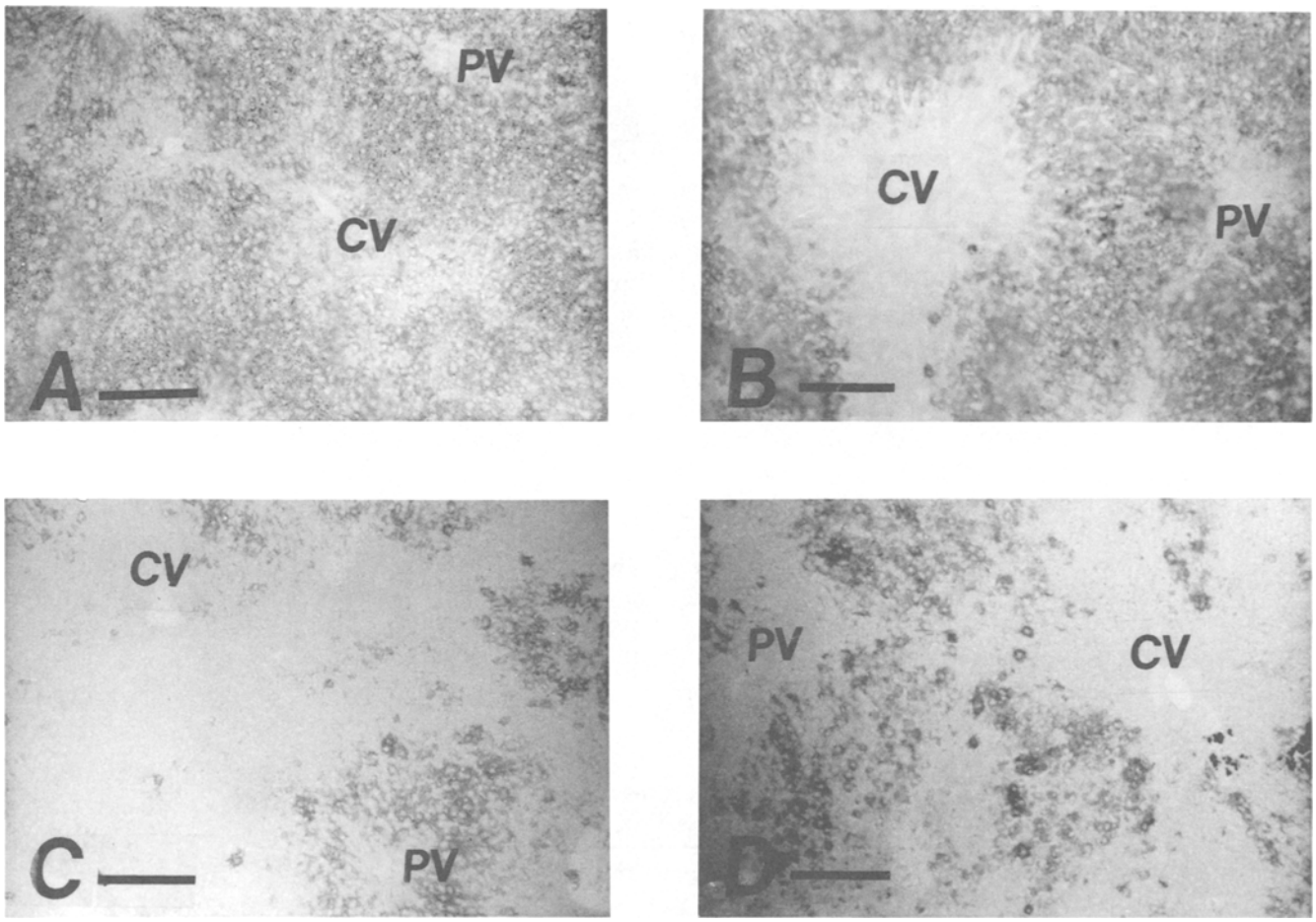


FIG. 2. Typical photomicrographs of liver sections stained with hematoxylin and Oil Red. A, control diet; B, control + PCB diet; C, highly hydrogenated soybean oil (HSO)-A diet; D, HSO-A + PCB diet. CV and PV in the photographs mean central vein and portal vein, respectively. Scale in the photographs indicates 200 μm (reduced by 50%). See Figure 1 for other abbreviation.

TABLE 5
Effects of Dietary PCBs and Highly Hydrogenated Soybean Oil on the Fatty Acid Composition of Rat Hepatic Phospholipids[a]

Fatty acid	Control diet (%)	Control + PCBs diet (%)	HSO-A diet (%)	HSO-A + PCBs diet (%)
14:0	0.4 ± 0.01	0.4 ± 0.01	0.5 ± 0.01	0.4 ± 0.01
16:0	12.8 ± 0.15[a]	11.9 ± 0.08[b]	10.6 ± 0.09[c]	10.5 ± 0.15[c]
16:1	0.4 ± 0.01	0.4 ± 0.01	0.5 ± 0.01	0.4 ± 0.01
18:0	21.6 ± 1.01[a]	24.9 ± 1.20[b]	25.3 ± 1.11[b]	28.8 ± 1.22[c]
18:1	8.9 ± 0.68[a]	7.7 ± 0.48[b]	9.0 ± 0.77[a]	7.3 ± 0.42[b]
18:2	14.0 ± 0.25[a]	13.7 ± 0.26[a]	12.6 ± 0.66[b]	10.5 ± 0.77[c]
18:3	0.3 ± 0.01	0.3 ± 0.01	0.3 ± 0.01	0.3 ± 0.01
20:0	0.2 ± 0.03	0.2 ± 0.02	0.1 ± 0.03	0.1 ± 0.05
20:4	29.8 ± 0.66[a]	30.9 ± 0.45[a,b]	29.4 ± 0.53[a]	32.0 ± 0.66[b]
20:5	0.2 ± 0.02[a]	n.d.	0.3 ± 0.01[b]	n.d.
22:0	0.4 ± 0.11	0.2 ± 0.13	0.2 ± 0.11	0.3 ± 0.10
22:1	1.7 ± 0.21[a]	1.4 ± 0.18[a]	2.8 ± 0.28[b]	1.5 ± 0.14[a]
22:6	9.2 ± 0.42[a]	8.1 ± 0.49[b,c]	8.7 ± 0.33[a,b]	8.0 ± 0.32[c]
SFA	35.5 ± 0.46[a]	37.6 ± 0.55[b]	36.6 ± 0.59[b]	40.1 ± 0.75[c]
MUFA	11.1 ± 0.65[a]	9.5 ± 0.45[b]	12.3 ± 0.79[a]	9.1 ± 0.52[b]
n-6 PUFA	43.8 ± 0.56[a]	44.6 ± 0.62[a]	42.0 ± 0.51[b]	42.5 ± 0.68[b]
n-3 PUFA	9.6 ± 0.41[a]	8.4 ± 0.48[b,c]	9.2 ± 0.41[a,b]	8.3 ± 0.37[c]
P/S	1.8 ± 0.34	1.7 ± 0.36	1.7 ± 0.45	1.5 ± 0.36
n-6/n-3	4.5 ± 0.36[a]	5.3 ± 0.38[b]	4.6 ± 0.33[a]	5.1 ± 0.35[a,b]

[a]Each value shows mean ± SEM, $n = 6$. In each experiment, values in the same line without common superscript letters denote significant difference ($P < 0.05$). n.d. = not detected. The abbreviations, SFA, MUFA, and PUFA mean saturated fatty acid, monounsaturated fatty acid and polyunsaturated fatty acid, respectively. P/S means PUFA/SFA. See Tables 1 and 3 for other abbreviations.

tered along the distribution of lipids stained by Oil Red (Fig. 2). This indicated that dietary PCBs injured the area around the central vein. A decrease in the level of liver lipids by dietary HSO would be in concert with the lack of Oil Red staining in livers from rats on the HSO diets.

The changes of fecal neutral sterols, namely the disappearance of coprostanol, were observed in the feces in rats given the HSO diet (Table 4), suggesting a shift in enterobacteria, for example, a decrease in the population of bacteria such as *Eubacterium* having a specific enzyme that reduces cholesterol to coprostanol (27), although we did not perform bacteriological examinations.

As shown in Table 5, HSO ingestion increased the 18:0 content of hepatic phospholipid, suggesting that 18:0 in HSO was partly absorbed and contributed to the increase in the proportion of saturated fatty acid content. Actually, about 43% of triacylglycerol in HSO was distearoylmonoacylglycerol whose absorption was considered relatively easy. PCB ingestion also increased the 18:0 proportion of the fatty acid content in hepatic phospholipid. These findings might be the result of the activation of fatty acid synthesis, because dietary PCBs are known to increase lipid synthesis (28). The supply of 18:0 by dietary HSO and the increase of this fatty acid by PCBs were independent, and caused a 7% increase in the stearic acid ratio and a 4.6% increase in total saturated acid in the hepatic phospholipids of rats given HSO-A + PCBs compared with controls. The increased saturated fatty acid proportion seemed to affect other physiological situations like the fluidity of membrane lipids.

In contrast to 18:0, 18:1 was reduced by about 2% by dietary PCBs, but not by dietary HSO. According to Leikin and Brenner (29), Δ^9-desaturase is stimulated by dietary cholesterol, which also increases the 18:1 ratio accompanied with a decrease in the proportion of saturated fatty acids. This mechanism was explained by an altered fluidity of membrane lipids. Whether the alteration of Δ^9-desaturase activity is associated with a specific response to PCBs or the different origin of the cholesterol supply remains to be ascertained.

With respect to the n-6 polyunsaturated fatty acid levels, the concentration of 18:2 decreased, with a reciprocal increase of the 20:4 proportion in rats given PCBs regardless of HSO in the diet. Borlakoglu *et al.* (30) observed the increase of fatty acid desaturation in rat hepatic microsomes at 5 d after intraperitoneal injection of PCBs. Similarly, dietary PCBs might stimulate the Δ^6- and Δ^5-fatty acid desaturases to alter the ratio of 18:2 and 20:4. As 20:4 is relevant to inflammation, PCBs-mediated increase of this fatty acid content in hepatic phospholipid might cause some undesirable physiological or pathological situations. We have shown in this study that insofar as those situations are concerned, dietary HSO would not be effective because it cannot prevent the increase of the 20:4 content. Also, dietary HSO could modify some PCBs-mediated physiological changes including the alteration of liver and fecal lipids, and the accumulation of PCBs in the liver.

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Dietary Docosahexaenoic Acid Has Little Effect on Peroxisomes in Healthy Mice

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ABSTRACT: NMRI mice were fed diets supplemented with 0.05, 0.2, or 2% (w/w) docosahexaenoic acid (DHA), a polyunsaturated fatty acid present in fish oil, for 3 d, 3 wk, or 3 mon. The doses of DHA were chosen to supply the mice with concentrations of DHA which approximate those that have been reported to be beneficial to patients with peroxisomal disease. Diets containing 0.05 or 0.2% DHA did not change hepatic, myocardial, and renal catalase (EC 1.11.1.6) activity except for a slight but significant increase (to 120%) in myocardial catalase activity in mice treated with the 0.05% DHA diet for 3 mon. A diet with 2% DHA induced myocardial catalase activity to 150% after both 3 d and 3 wk of administration. In the liver of mice fed this diet for 3 wk, hepatic catalase activity was increased to 140% while no induction of palmitoyl-CoA oxidase (EC 1.3.99.3), urate oxidase (EC 1.7.3.3), and L-α-hydroxyisovalerate oxidase (EC 1.1.3.a) was observed. With the light microscope, no changes in peroxisomal morphology were visually evaluated in catalase stained sections of liver, myocardium, and kidney of mice fed either diet. Our results show that in healthy mice a low dietary DHA dose (<0.2%; this corresponds to a dose prescribed to peroxisomal patients) has no effect on several hepatic peroxisomal H_2O_2-producing enzymes, including the rate-limiting enzyme of the peroxisomal fatty acid β-oxidation. This may indicate that such a DHA dose will not add a strong load on the often disturbed fatty acid metabolism in the liver of patients with peroxisomal disorders.

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Peroxisomes are cell organelles characterized by the presence of H_2O_2-producing oxidases and high amounts of catalase, a H_2O_2-scavenging enzyme (1). These and other peroxisomal enzymes take part in several metabolic processes, thus playing a variety of critical roles in cellular metabolism. A deficiency of only one peroxisomal enzyme may already induce a spectrum of severe clinical symptoms (2). Today there exists no established therapy for patients with a deficiency of one or more peroxisomal enzymes.

Based on the finding that patients with several peroxisomal disorders show too low levels of docosahexaenoic acid (DHA) in several organs (3,4), it has been suggested to supplement this polyunsaturated fatty acid in the diet of peroxisomal disorder patients (5,6). While in some clinical trials fish oil is added to the diets of patients with peroxisomal disorders (7), others recommend the use of purified DHA (5,8). Clinical improvement of patients treated with dietary DHA supplementation was recently reported (5,8).

We previously studied the effect of different doses of a fish oil extract on the peroxisomes in liver, myocardium, and kidney of healthy mice (9,10). The investigated fish oil doses were chosen in such a way that the daily amount of DHA administered to the mice approximated the doses which were beneficial to patients with a peroxisomal disease. An increase in peroxisomal β-oxidation and catalase activity in liver and myocardium was observed but only when doses higher than the doses used in clinical settings were administered (9–11).

The aim of this study was to investigate the effect of dietary supplementation of purified DHA on peroxisomes in mice. The studied DHA doses were identical to the DHA doses previously selected in the fish oil experiments (9–11). As this study was being completed, data on the effect of DHA supplementation to rats were published (12,13). Preliminary results have been reported during the Sixth Belgian Week of Gastroenterology; Knokke, Belgium, March 16–18, 1994 (14).

MATERIALS AND METHODS

Adult male NMRI mice (35–40 g) were randomly assigned into groups of at least five animals. They were fed a standard laboratory meal (A04; UAR, Epinay, France) supplemented with 0.05% DHA ethyl ester for 3 d, 3 wk, and 3 mons or with 0.2 or 2% DHA ethyl ester for 3 d and 3 wk. Ethyl DHA was obtained from Sigma (Bornem, Belgium). DHA is a polyunsaturated fatty acid present as a main compound in fish oil extracts. The composition of the standard laboratory meal has previously been given (10). The dietary DHA doses were chosen to approximate the daily oral dose of 250 mg DHA ethyl ester administered in a clinical setting to children with generalized peroxisomal disorders (5,8). We considered the body surface area as reference point to convert doses from man to mouse (15). To express a mg/kg dose in any given species as the equivalent mg/m^2 dose, the dose has to be multiplied by the appropriate ratio between surface area and body weight. This ratio is 3 for the mouse and is 25 for a child of 20 kg

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Abbreviation: DHA, docosahexaenoic acid (C22:6n-3).

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(15). We calculated that a dose of 250 mg/d given to a child with a maximum body weight of 20 kg (which is in fact a daily intake of 12.5 mg/kg body weight) corresponds to a daily intake of 12.5 × 25/3 = 104 mg/kg body weight in the mouse. The mean intake of DHA by the mice on a diet with 0.05, 0.2, or 2% DHA was 87, 350, and 3500 mg/d/kg body weight, respectively, being slightly smaller to approximately 3.5- and 35-fold the clinical dose. The time control groups received the A04 meal only. All animals had free access to food and water. The appetite of the animals under DHA treatment was not different from control animals.

The activities of peroxisomal fatty-acyl CoA oxidase (EC 1.3.99.3), urate oxidase (EC 1.7.3.3), L-α-hydroxy acid oxidase (EC 1.1.3.a) and D-amino acid oxidase (EC 1.4.3.3) were assayed in liver homogenates of mice treated with the highest DHA-supplemented diet (2%) for 3 wk using the method of Osumi and Hashimoto (16) with the following substrates: palmitoyl-CoA, urate, L-α-hydroxyisovalerate, D-alanine, and D-proline, respectively. Catalase (EC 1.11.1.6) activity was assayed in homogenates of liver, heart, and kidney cortex according to Baudhuin (17,18).

Histological analysis (after diaminobenzidine staining for catalase) of the peroxisomes in liver, myocardium, and kidney were performed as previously described (19). Results are given as the mean ± SD. For statistical analysis, the Mann-Whitney test was used (20). Significance was obtained when $P < 0.05$.

RESULTS

The DHA diet was well accepted by the mice. At the end of the experiment, body weight of DHA-treated animals was similar to the weight of control animals. No hepatomegaly was observed. Liver, heart, and kidney weights expressed as percentages of body weight were not changed (results not shown). The time control groups showed no differences in catalase activity and histological appearance of the peroxisomes and, therefore, were pooled into one control group.

Hepatic catalase activity was not altered in mice fed a diet supplemented with 0.05 or 0.2% DHA when compared to the activity in control mice (76 ± 6 U_B/g liver) (Fig. 1). A significant increase in catalase activity (to 140%) was observed in mice fed the highest DHA dose for 3 wk. Treatment for 3 d had no effect on catalase activity (Fig. 1). In myocardium of mice fed a diet supplemented with 0.05% DHA, only the prolonged treatment of 3 mon induced a slight but significant increase in catalase activity (to 120%) when compared to control animals (1.26 ± 0.10 U_B/g myocardium) (Fig. 2). Feeding diets with 0.05 or 0.2% DHA for 3 d or 3 wk had no effect on myocardial catalase activity. An increase to ± 150% of control catalase activity was found in mice fed 2% DHA-supplemented diet for 3 d and 3 wk (Fig. 2). Renal catalase activity was not altered in mice fed a DHA-containing diet for up to 3 wk (2% DHA-diet) or for up to 3 mon (0.05% DHA-diet) (Fig. 3).

The activities of several H_2O_2-generating peroxisomal oxidases were assayed in the liver of mice with a significant increase in hepatic activity of the H_2O_2-scavenging peroxisomal enzyme, catalase. Palmitoyl-CoA oxidase, urate oxidase, and L-α-hydroxyisovalerate oxidase activities were not changed in animals fed the 2% DHA-diet for 3 wk (Table 1). No activity of D-amino acid oxidase using D-proline and D-alanine as a substrate was detected in both mice fed a control or a DHA-containing diet.

Light microscopic study of diaminobenzidine-stained sections of liver, myocardium, and kidney revealed the presence of peroxisomes in both control and DHA-treated mice. In the liver, peroxisomes were observed throughout the parenchyma. No alterations in staining intensity, size, number, or distribution of the peroxisomes in DHA-treated animals were observed (Fig. 4). Myocardial peroxisomes revealed a heterogeneous distribution in all investigated samples being apparently absent

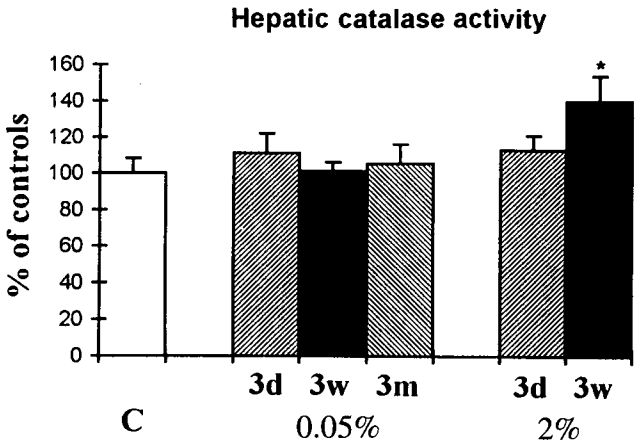


FIG. 1. Catalase activity in liver homogenates of mice treated with 0.05% DHA and 2% DHA for up to 3 mon. Significant increases from control data (C) (76 ± 6 U_B/g liver = 100%) are indicated with *.

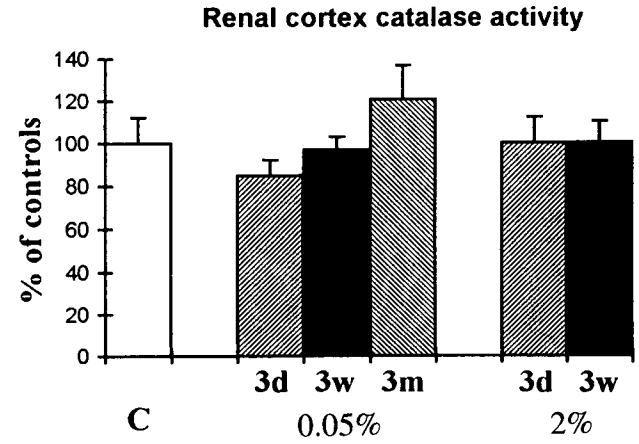


FIG. 2. Catalase activity in renal cortex homogenates of mice treated with 0.05% DHA and 2% DHA for up to 3 mon. No significant changes from control data (C) (33 ± 2 U_B/g kidney cortex = 100%) are observed.

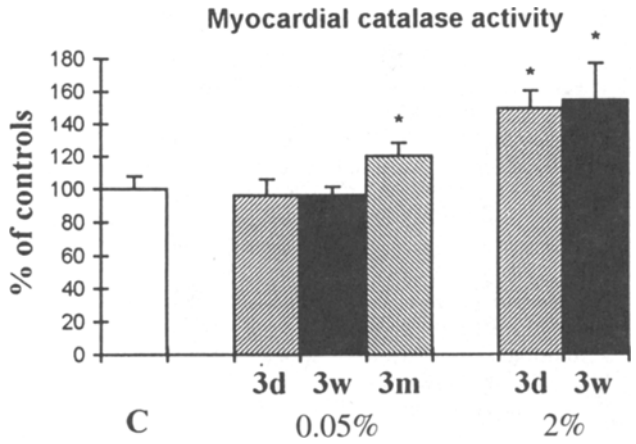


FIG. 3. Catalase activity in myocardium homogenates of mice treated with 0.05% DHA and 2% DHA for up to 3 mon. Significant increases from control data (C) (1.26 ± 0.10 U_B/g myocardium = 100%) are indicated with *.

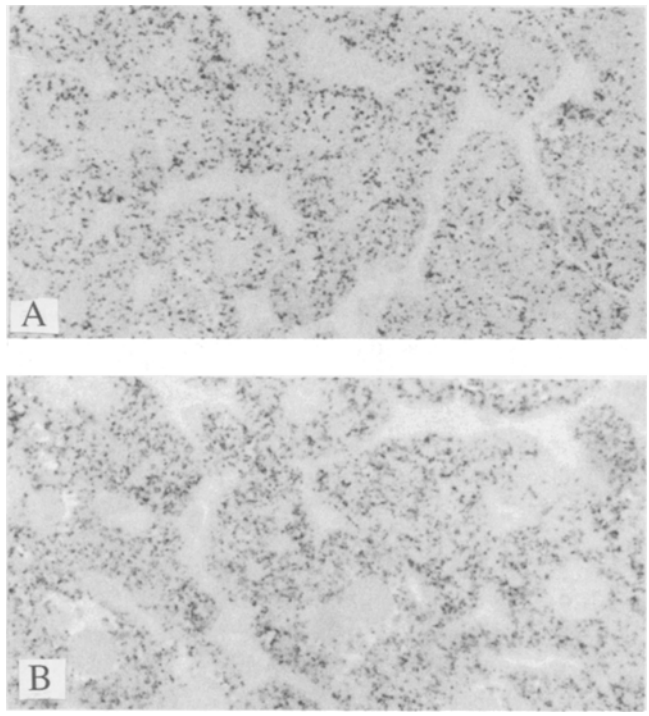


FIG. 4. Light micrographs of liver of mice treated with a control diet (A) or a diet with 2% DHA (B). Liver sections were stained for catalase activity. Catalase reaction product is found in the peroxisomes which are recognized as dots in the cytoplasm. No changes in size, shape, and distribution of the peroxisomes are observed. Catalase cytochemistry. X 500.

TABLE 1
Peroxisomal Oxidase Activities in Liver Homogenates of Mice Treated with 2% DHA (for 3 wk)[a]

Diet	*n*	Palmitoyl-CoA oxidase	Urate oxidase	L-α-hydroxyisovalerate oxidase
Control	5	1.221 ± 0.201	1.371 ± 0.456	0.848 ± 0.055
DHA	5	1.391 ± 0.244	1.110 ± 0.290	0.818 ± 0.081

[a]Enzyme activities are expressed as nmol H_2O_2/min/mg liver; DHA, docosahexaenoic acid.

in some fibers and numerous in adjacent fibers. This characteristic made it difficult to visually evaluate changes in number and distribution of the peroxisomes. In DHA-treated mice, size and staining intensity of myocardial peroxisomes were similar to those observed in control mice. Renal peroxisomes were predominantly observed in the epithelium of proximal tubules. No differences in morphological characteristics of the peroxisomes in proximal tubules between DHA-treated and control mice were observed. A few small peroxisomes were infrequently observed in the glomeruli, distal tubules, and collecting tubules in all investigated renal sections.

DISCUSSION

We found that a 3 wk administration of 2% DHA (which corresponds to a daily uptake of 3500 mg DHA/kg body weight) induced catalase activity in mouse liver while no induction of three peroxisomal oxidases (fatty acyl-CoA oxidase, urate oxidase, and L-α-hydroxyacid oxidase) was provoked. Lower doses (corresponding to a daily uptake of 87 and 350 mg DHA/kg body weight) enclosing the doses prescribed to patients with peroxisomal diseases did not change hepatic catalase activity. In rats that received 1000 mg DHA/d/kg body weight by gastric intubation for 3 mon, a significant induction of hepatic catalase activity (to 118%) was observed (13). This dose corresponds to 500 mg DHA/d/kg body weight in the mouse when the body surface area is considered as reference point to convert doses between rats and mice (15). In contrast to our results, a significant increase in peroxisomal fatty acyl-CoA oxidase activity (to 164%) was observed in these rats; this effect was also obtained in rats treated with 500, 1000, and 1500 mg DHA/d/kg body weight by gastric intubation for 10 d (12). The discrepancy in results may be ascribed to differences in DHA administration, type of animals used, and composition of the diets.

Mice fed the 2% DHA diet for 3 wk revealed a three-times smaller induction of hepatic catalase activity when compared to mice of the same strain, age, and sex fed the 10% fish oil diet that contains the same final concentration of DHA (9). In addition peroxisomal β-oxidation was increased at least 6.6-fold in the mice fed 10% fish oil while no induction was found in mice treated with 2% DHA diets. These data suggest that in mice the induction of peroxisomal enzymes in the liver can mainly be ascribed to the high concentration of fatty acids in the fish oil diet. This assumption also holds true for the inducing effects of other high-fat diets which often have a composition totally different from fish oil (9).

Myocardial catalase activity was induced in mice fed the 2% DHA-containing diet. We previously reported a stronger

induction of catalase activity in mice fed a 10% fish oil diet (in which the concentration of DHA is 2%) (9,10). This suggests that part of the catalase activity induction observed in myocardium of mice treated with dietary fish oil may be ascribed to the presence of DHA. No data on catalase activity in myocardium of treated rats are available (12,13).

To our knowledge, data on the effects of dietary supplementation of DHA, alone or as part of a fatty acid mixture other than fish oil, on the functions and morphology of renal peroxisomes are lacking. We found no changes in catalase activity, in intensity of peroxisomal catalase staining, and in size, shape, and distribution of peroxisomes in the kidney of mice fed either diet. This is in line with our previous findings made in mice fed fish-oil supplemented diets (9,10).

The increases in catalase activity observed in liver and myocardium are not reflected in an enhanced staining intensity of the individual peroxisomes. This indicates that the intensity of the oxidized diaminobenzidine in sections cytochemically stained for catalase is little susceptible to the observed changes in activity of this enzyme.

Our results indicate that dietary supplementation of DHA in doses approximating the daily amount of DHA prescribed to patients with peroxisomal disorders has little effect on the peroxisomal compartment in healthy mice. Only myocardial catalase activity was increased in long-term (3 mon) treated animals and suggests the need for an increased H_2O_2-scavenging activity. However, in liver of mice treated with 2% DHA, the increased catalase activity was not related to an induction of three H_2O_2-producing enzymes including peroxisomal fatty acyl-CoA oxidase. An increased H_2O_2 pool may be brought on by other peroxisomal oxidases or by the import of extraperoxisomal H_2O_2 as the peroxisomal membrane is permeable for small solutes (21). A strong increase in peroxisomal β-oxidation without an as strong induction of catalase activity is a general phenomenon observed in rodents treated with peroxisome proliferators, a heterogeneous group of drugs and chemical substances (22). Several members of this group of products show an increased incidence of hepatic tumors in long-term treated rodents. This characteristic has been ascribed to the disturbed equilibrium between H_2O_2-generating and H_2O_2-scavenging pathways.

We conclude that DHA doses which gave clinical improvement to patients with generalized peroxisomal disorders are low enough not to be a burden for β-oxidation in liver and kidney of healthy mice. Only the unknown immediate cause for induction of catalase activity in myocardium of mice treated for 3 mon with the low dose of DHA could be a source of concern and needs further investigation.

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Modification of Membrane Fatty Acid Composition, Eicosanoid Production, and Phospholipase A Activity in Atlantic Salmon *(Salmo salar)* Gill and Kidney by Dietary Lipid

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ABSTRACT: Atlantic salmon post-smolts were fed diets containing either fish oils (Fosol, FO and Marinol, MO) rich in long-chain n-3 polyunsaturated fatty acids (PUFA), or plant oils rich in 18:2n-6 (sunflower oil, SO) or 18:3n-3 (linseed oil, LO) for 12 wk. The major PUFA in individual phospholipids from gill and kidney were related to the dietary lipid intake. Levels of n-6 PUFA were highest while levels of n-3 PUFA were lowest in fish fed SO. Fish fed LO generally had lower levels of 20:4n-6 compared to the other treatments while fish fed SO generally had the highest levels of 20:4n-6. In all phospholipid classes except phosphatidylinositol (PI) 20:5n-3 was greatest in fish fed MO followed by FO, LO, and SO. In PI, 20:5n-3 was also highest in fish fed MO but those fed LO contained more 20:5n-3 than those fed FO. This resulted in the ratio of the eicosanoid precursors, 20:4n-6/20:5n-3, being significantly greater in fish fed SO, for all phospholipid classes, compared to fish fed the other three dietary oils. The activity of gill phospholipase A was greatest in fish fed FO and was lowest in fish fed SO. The concentration of $PGF_{3\alpha}$ was significantly increased in gill homogenates from fish fed MO compared to the other three treatments while $PGF_{2\alpha}$ was significantly increased in fish fed SO compared to those fed LO. The concentration of PGE_3 was significantly reduced in kidney homogenates from fish fed SO compared to the other three treatments while PGE_2 was significantly increased in fish fed SO compared to those fed either FO or LO.
Lipids 31, 1163–1171 (1996).

Considerable evidence now exists to suggest that overproduction of 2-series eicosanoids, derived from arachidonic acid (20:4n-6, AA), is fundamental in increased occurence of a number of chronic inflammatory diseases in humans, particularly cardiovascular and autoimmune conditions (1,2). Evidence that the long-chain n-3 polyunsaturated fatty acids (PUFA) present in fish oils, namely eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), have an ameliorating effect on the above conditions is well documented (3,4). EPA can be metabolized by both lipoxygenase and cyclooxygenase to yield a range of eicosanoids although their biological efficacy is generally reduced compared to their AA-derived analogues (5). Additionally, both EPA and DHA, and α-linolenic acid (18:3n-3) can inhibit cyclooxygenase activity and thus reduce production of eicosanoids derived from AA (6,7).

The rate and type of eicosanoid synthesis, at the cellular level, depend on the size and composition of the available precursor pool which consequently can be modulated by dietary PUFA intake (8). At least three enzymes, Δ6- and Δ5-desaturase (9) and phospholipase A_2 (10), are involved in supply of precursor PUFA for eicosanoid synthesis, and the reactions they catalyze can be influenced by a number of nutritional and physiological factors (9,11). Thus, in addition to their ability to inhibit eicosanoid production directly, n-3 PUFA can, by inhibition of linoleic acid desaturation and elongation, further reduce the synthesis of 2-series eicosanoid production (12).

Fish offer a useful model system for investigating the synthesis and interaction of eicosanoids of the 2- and 3-series since their tissues are naturally abundant in both EPA and DHA (13,14). However, despite the apparent excess of EPA over AA in their phospholipids, fish do produce considerable quantities of AA-derived eicosanoids (15), and a number of studies suggest that AA is the preferred substrate for cyclooxygenase in plaice neutrophils, turbot gills, and brain astroglial cells in culture (16–18). In previous studies, Atlantic salmon given diets containing 18:2n-6 or 18:3n-3 have shown increased levels of AA and EPA, respectively, which have influenced the production of AA-derived eicosanoids (19, 20). The ability of the lipoxygenase product 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) to modulate the activity of ion channels has been observed in mammals (21), and clearly this activity would be important in gills which,

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Abbreviations: AA, arachidonic acid; ANOVA, analysis of variance; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FO, Fosol fish oil; HETE, hydroxy eicosatetraenoic acid; HPTLC, high-performance liquid chromatography; LO, linseed oil; MO, Marinol fish oil; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PGE, prostaglandin E; PGF, prostaglandin F; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SO, sunflower oil; TLC, thin-layer chromatography.

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along with the posterior kidney are vital in osmoregulation in fish. The ability of fish gill and kidney to synthesise prostaglandins has been known for some time although their functions were originally not clear (22,23). More recently prostaglandin E (PGE_2) and PGE_1 have been identified as active antidiuretic agents in trout and eel kidney (24,25) while PGE_2 inhibits ion transport in gill epithelia (26). Clearly diet-induced changes in gill and kidney eicosanoid products might affect the ability of salmon to osmoregulate effectively which would compromise their ability to adapt to the marine environment when undergoing parr-smolt transformation.

In the present experiment, four groups of Atlantic salmon were given diets enriched in either 18:2n-6 (sunflower oil), 18:3n-3 (linseed oil), or 20:5n-3 (Marinol, southern hemisphere fish oil) which were compared to a control group fed a northern hemisphere fish oil (Fosol). The phospholipid fatty acid compositions of gill and kidney were determined along with concentrations of prostaglandins PGE and PGF of the 2- and 3-series and gill phospholipase A activity.

MATERIALS AND METHODS

Fish and husbandry. Four hundred Atlantic salmon S1 smolts of mean weight 54 g were obtained from the Scottish Office Agriculture Environment and Fisheries Department (S.O.A.E.F.D.) Fish Cultivation Unit (Aultbea, Wester Ross, Scotland) and distributed randomly into four tanks of 2000 L each, and supplied with nonrecirculated seawater at a rate of 26 L/min. The fish were subject to natural photoperiod, and the water temperature during the experimental period (August–November) varied from 15–9°C. Diets were supplied by automatic feeders which were adjusted to supply 30 g/kg biomass per day, until the average fish weight reached 100 g, and thereafter this was reduced to 20 g/kg biomass for the remainder of the experiment. The feeding trial was conducted for a period of 3 mon, and fish were weighed individually at the start and finish of the trial and weighed in bulk at 28-d intervals after which the ration was adjusted accordingly. The experiment was conducted in accordance with British Home Office guidelines regarding research on experimental animals.

Diets. The composition of the basal diet has been described in detail previously (27) and utilized a low-temperature fishmeal as protein source. The lipid component of 100 g/kg was either Fosol (FO), a Northern hemisphere fish oil derived from pelagic fish, mainly herring, capelin and sand eel (Seven Seas Ltd., Hull, United Kingdom), sunflower oil (SO) (Tesco Ltd, Cheshunt, United Kingdom), linseed oil (LO) (Omega Nutrition Inc., Vancouver, Canada), or Marinol (MO), a Southern hemisphere fish oil derived from sardines, pilchards, and anchovies (Fishing Industry Research Unit, Rosebank, 7700 Republic of South Africa). An antioxidant mix was added to the oils before mixing with the dry ingredients (27). The diets were formulated to satisfy the nutritional requirements of salmonid fish (28) and contained 46% crude protein and 16% lipid. The fatty acid compositions of the four experimental diets are shown in Table 1.

TABLE 1
Fatty Acid Compositions of Diets Containing (wt%) Fosol (FO), Sunflower Oil (SO), Linseed Oil (LO), and Marinol K (MO) Fed to Atlantic Salmon Post Smolts

Fatty acid	FO diet	SO diet	LO diet	MO diet
14:0	5.5	1.8	1.8	6.7
16:0	14.0	9.7	8.2	15.9
18:0	2.2	3.0	2.6	2.6
Total saturates[a]	22.2	15.2	12.7	26.0
16:1n-7	3.9	2.6	1.8	6.5
18:1n-9	11.8	16.2	16.5	10.2
18:1n-7	3.2	2.5	2.2	3.3
20:1n-9	9.9	4.4	3.9	6.4
22:1n-11	12.7	3.5	3.7	7.8
24:1	1.1	0.3	0.4	0.7
Total monoenes	44.2	30.5	28.7	35.9
18:2n-6	1.5	40.2	9.7	1.5
20:2n-6	0.3	0.5	0.5	0.5
20:3n-6	—	—	—	trace
20:4n-6	0.5	0.2	0.2	0.7
22:5n-6	0.2	trace	trace	0.2
Total n-6[b]	2.5	40.9	10.4	3.1
18:3n-3	1.4	0.7	34.8	1.3
18:4n-3	2.8	0.8	0.9	2.4
20:4n-3	0.8	0.2	0.2	0.7
20:5n-3	6.6	3.2	2.8	13.1
22:5n-3	1.2	0.3	0.3	1.4
22:6n-3	11.5	5.1	5.2	11.5
Total n-3	24.3	10.3	44.3	30.4
Total PUFA[c]	30.0	51.4	54.7	36.1
n-3/n-6	9.7	0.3	4.3	9.8
20:4/20:5	0.08	0.06	0.03	0.05

[a]Includes 15:0, 17:0, and 20:0.
[b]Includes 18:3n-6 and 22:4n-6.
[c]Polyunsaturated fatty acids include 16:2, 16:3, and 16:4; trace value <0.05%; — = not detected. Fosol is a Northern hemisphere fish oil derived from pelagic species, herring, capelin, and sand eel. Marinol is a Southern hemisphere fish oil derived from sardine, pilchard, and anchovy.

Extraction and analysis of lipids. Intact gill arches and posterior kidney samples were dissected from four fish per dietary treatment and immediately frozen in liquid nitrogen. Samples were stored at –40°C prior to lipid extraction. Total lipid was extracted from gill, kidney, and diet samples by the method of Folch *et al.* (29). The details of extraction, separation of phospholipid classes, and preparation of fatty acid methyl esters have been described in detail previously (27). The fatty acid methyl esters were separated and quantified by gas–liquid chromatography (Carlo Erba Vega 6000; Fisons Ltd., Crawley, United Kingdom) using a 50 m × 0.22 mm capillary column (free fatty acid phase, S.G.E. Ltd., Milton Keynes, United Kingdom). Hydrogen was used as carrier gas and temperature programming was from 50 to 160°C at 39°C/min and then to 225°C at 2°C/min. Individual methyl esters were identified by comparison to known standards and by reference to published data (30).

Extraction, separation, and enzyme immunoassay of PGE and prostaglandin F (PGF) isomers. Gill and kidney samples were dissected out, weighed, and immediately homogenized

in 3 mL of Hank's balanced salt solution containing 15% ethanol (vol/vol) and 0.15 mL of 2M formic acid. Homogenates were frozen in liquid nitrogen and stored at −20°C until the eicosanoids were extracted. The frozen homogenates were thawed and centrifuged (1000 × *g*, 5 min) to precipitate cell debris. The supernatants were extracted using octadecyl silica (C18) Sep-Pak minicolumns [Millipore (United Kingdom), Watford, United Kingdom)] by the method of Powell (31) and as described in detail by Bell *et al.* (18). The final extract was dissolved in 0.1 mL of methanol prior to HPLC. The isomers were separated by reverse phase high-performance liquid chromatography (HPLC) largely as described in Bell *et al.* (18). An isocratic solvent system was employed containing 17 mM phosphoric acid/acetonitrile (70:30, vol/vol) at a flow rate of 1 mL/min. The elution times of the two PGE and two PGF standards were determined by ultraviolet (UV) detection at 196 nm using a Pye-Unicam LC-UV detector. Fifty μL of the eicosanoid homogenate extract was injected onto the column and 1 mL fractions were collected using an LKB 2112 Redirac. Fractions corresponding to each PGE isomer were pooled and extracted as follows. The pooled fractions were applied to a C18 Sep-Pak which had been pre-washed with 5 mL methanol and 10 mL distilled water. The column was then washed with a further 10 mL of distilled water, and then the PGE eluted in 5 mL ethyl acetate. Samples were dried under nitrogen and redissolved in immunoassay buffer. The remaining 50 μL of the eicosanoid extract was injected onto the column, and fractions corresponding to the two PGF isomers were collected and concentrated as described for PGE isomers. Measurement of PGE and PGF isomers of the 2- and 3-series was performed using enzyme immunoassay kits for PGE_2 and $PGF_{2\alpha}$ according to the manufacturers' protocols (SPI-Bio, Massy, France). Cross reactivity of PGE_3 for the PGE_2 antibody and of $PGF_{3\alpha}$ for the $PGF_{2\alpha}$ antibody was 100% in both cases.

Determination of phospholipase A activity. Phospholipase A activity was assayed with endogenous substrate largely as described by Szymanska *et al.* (32). Gill filaments were dissected from the gill arch and homogenized in 2 mL of 50 mM Tris-HCl (pH 8.5) containing 200 mM KCl and $CaCl_2$. One mL was removed and the lipid extracted immediately by the method of Folch *et al.* (29), 0.1 mL was removed for protein determination and the remaining homogenate placed in a shaking water bath at 18°C. After 2 h, the reaction was terminated by addition of 5 mL of chloroform/methanol (2:1, vol/vol) containing 0.05% butylated hydroxytoluene and the lipids extracted as described above in the *Extraction and analysis of lipids* section. Free fatty acids, phosphatidylethanolamine (PE), and phosphatidylcholine (PC) in 0- and 2-h samples were separated by double-development high-performance thin-layer chromatography (HPTLC) as described by Bell *et al.* (19) and quantified by scanning densitometry using a Shimadzu CS-9000 (Tokyo, Japan) dual wavelength TLC scanner and DR-13 recording integrator.

Materials. TLC plates (20 × 20 cm × 0.25 mm) and HPTLC plates (10 × 10 cm × 0.25 mm) pre-coated with silica gel 60 were obtained from Merck (Darmstadt, Germany). All solvents were HPLC grade and were obtained from Rathburn Chemicals Ltd. (Walkerburn, United Kingdom). Hank's balanced salt solution was obtained from Flow Ltd. (Rickmansworth, United Kingdom).

Statistical analysis. Significance of difference ($P < 0.05$) between dietary treatments was determined by analysis of variance (ANOVA). Analyses were performed using a Statgraphics (system 3.0) computer package (Statistical Graphics Corporation, Rockville, MD). Data which were identified as nonhomogeneous (using Bartlett's test) were subjected to either arcsine square root or log transformation before ANOVA. Differences between means were determined by Tukey's test.

RESULTS

The fatty acid compositions of gill phospholipid classes are shown in Tables 2–5. In PC (Table 2) the level of total monoenoic fatty acids was significantly reduced in fish fed SO compared to those fed FO. Linoleic acid content was significantly different for each dietary treatment with the highest level in SO-fed fish and the lowest in those fed MO. Levels of 20:2n-6, 20:3n-6, and total n-6 PUFA were all significantly greater in fish fed SO compared to those fed LO which, in turn, had significantly higher levels than in either the FO- or MO-fed fish. AA was significantly higher in gill PC from fish fed SO compared to those fed any of the other diets but was significantly lower in fish fed LO compared to those fed FO. α-Linolenic acid, 18:3n-3, 20:3n-3, and 20:4n-3 were significantly increased in salmon fed LO compared to the other

TABLE 2
Fatty Acid Compositions of Phosphatidylcholine from Gill of Salmon Fed Either FO, SO, LO, or MO[a]

Fatty acid	FO diet	SO diet	LO diet	MO diet
Total saturates	30.1 ± 0.8	28.3 ± 1.5	28.3 ± 1.0	30.1 ± 0.9
Total monoenes	22.3 ± 0.8^{a}	18.6 ± 2.0^{b}	20.8 ± 2.0^{a,b}	21.6 ± 1.2^{a,b}
18:2n-6	0.6 ± 0.1^{a}	9.8 ± 0.2^{b}	3.3 ± 0.1^{c}	0.3 ± 0.1^{d}
18:3n-6	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
20:2n-6	0.1 ± 0.0^{a}	1.4 ± 0.1^{b}	0.5 ± 0.1^{c}	0.1 ± 0.0^{a}
20:3n-6	0.2 ± 0.1^{a}	3.4 ± 0.6^{b}	1.1 ± 0.3^{c}	0.2 ± 0.0^{a}
20:4n-6	3.3 ± 0.1^{a}	4.7 ± 0.6^{b}	2.8 ± 0.2^{c}	3.2 ± 0.1^{a,c}
22:5n-6	0.6 ± 0.1	0.4 ± 0.0	0.6 ± 0.3	0.5 ± 0.1
Total n-6	4.8 ± 0.3^{a}	20.2 ± 0.2^{b}	8.6 ± 0.9^{c}	4.6 ± 0.2^{a}
18:3n-3	0.4 ± 0.0^{a}	0.2 ± 0.1^{a}	4.7 ± 1.0^{b}	0.3 ± 0.2^{a}
18:4n-3	0.2 ± 0.1^{a}	0.1 ± 0.0^{a}	0.5 ± 0.1^{b}	0.1 ± 0.1^{a}
20:3n-3	trace^{a}	trace^{a}	0.7 ± 0.1^{b}	trace^{a}
20:4n-3	0.5 ± 0.1^{a}	0.3 ± 0.1^{a}	1.8 ± 0.3^{b}	0.4 ± 0.1^{a}
20:5n-3	10.1 ± 0.5^{a}	5.5 ± 0.5^{b}	8.1 ± 0.4^{c}	12.5 ± 0.4^{d}
22:5n-3	1.5 ± 0.1^{a}	0.8 ± 0.1^{b}	1.3 ± 0.2^{a}	2.0 ± 0.1^{c}
22:6n-3	27.5 ± 0.8^{a}	23.2 ± 0.9^{b}	22.1 ± 1.4^{b}	26.0 ± 1.8^{a}
Total n-3	40.2 ± 0.6^{a}	30.1 ± 1.4^{b}	39.2 ± 0.9^{a}	41.3 ± 1.3^{a}
Total PUFA	45.0 ± 0.6^{a}	50.3 ± 1.6^{b}	47.9 ± 1.7^{b,c}	45.9 ± 1.3^{a,c}
n-3/n-6	8.4 ± 0.6^{a}	1.5 ± 0.1^{b}	4.6 ± 0.5^{c}	9.0 ± 0.6^{a}
20:4/20:5	0.3 ± 0.0^{a}	0.9 ± 0.1^{b}	0.4 ± 0.1^{a}	0.3 ± 0.1^{a}

[a]Values are weight % of total fatty acids. Values are means ± SD for four samples per dietary treatment. SD < 0.05 are tabulated as 0.0. Values in the same row with different superscript letters are significantly different ($P < 0.05$); trace value <0.05%. See Table 1 for abbreviations.

three treatments. EPA levels were significantly different in each dietary treatment with the highest value in fish fed MO followed by FO, LO, and lastly SO-fed fish. Levels of 22:5n-3 were significantly greatest in fish fed MO with those fed FO and LO having similar 22:5n-3 levels, while SO-fed fish had the lowest level. DHA levels were significantly greater in fish fed MO and FO compared to those fed SO or LO while total n-3 PUFA were significantly lower in SO-fed fish. Total PUFA were significantly greater in fish fed SO compared to those fed FO or MO and significantly greater in fish fed LO than fish fed FO. The n-3/n-6 PUFA ratio in fish fed FO and MO was significantly higher than in fish fed LO which was, in turn, significantly greater than those fed SO. The 20:4/20:5 eicosanoid precursor ratio was significantly greater in fish fed SO compared to the other three treatments.

The fatty acid compositions of gill PE are shown in Table 3. The level of total saturated fatty acids was significantly greater in fish fed MO compared to those fed LO. The levels of total monoenoic fatty acids were significantly different for each dietary treatment with the highest level in fish fed FO and the lowest in fish fed SO. The dietary induced changes in 18:2n-6, 20:2n-6, 20:3n-6, 22:5n-3, 20:4/20:5 ratio and total n-6 PUFA, in gill PE, were similar to those in PC, but AA was significantly elevated only in fish fed SO. α-Linolenic acid was significantly different in all dietary treatments with the highest level in fish fed LO and the lowest in fish fed SO while 20:3n-3 and 20:4n-3 were significantly increased only in fish fed LO. EPA was significantly increased in fish fed MO compared to all other treatments while FO- and LO-fed fish had significantly more EPA than those fed SO. Fish fed FO contained significantly more DHA than those fed MO, and both were significantly higher than those fed SO. Total n-3 PUFA were significantly greater in fish fed LO compared to all other treatments while those fed FO or MO contained significantly more n-3 PUFA than those fed SO. However, fish fed SO contained significantly more total PUFA than any other treatment, and LO-fed fish contained significantly more PUFA than either FO or MO-fed fish. The n-3/n-6 PUFA ratio was significantly different in all treatments with the highest value in MO-fed fish and the lowest in SO-fed fish. Total DMA were significantly greater in fish fed LO and MO compared to those fed SO and FO.

The fatty acid compositions of gill PI are shown in Table 4. Total saturates were significantly lower in fish fed FO compared to fish fed any of the other treatments. The level of 18:2n-6 was significantly greater in fish fed SO and significantly lower in those fed MO compared to all other treatments. The level of 20:2n-6 in gill PI was significantly greater in fish fed SO compared to those fed FO or MO. The level of 20:3n-6 was significantly greater in fish fed SO compared to the other three treatments and was significantly greater in fish fed LO compared to fish fed FO and MO. Total n-6 PUFA were significantly greater in fish fed SO compared to all other treatments. α-Linolenic acid and 20:3n-3 were significantly greater in gill PI from fish fed LO compared to the other three treatments while 20:3n-3 in fish fed MO was greater than in those fed FO or SO. The level of 20:4n-3 in fish fed LO was significantly greater than in fish fed SO or MO. EPA in gill PI was significantly greater in fish fed LO or MO compared to both other treatments while EPA was significantly lower in

TABLE 3
Fatty Acid Compositions of Phosphatidylethanolamine from Gill of Salmon Fed Diets Containing Either FO, SO, LO, or MO[a]

Fatty acid	FO diet	SO diet	LO diet	MO diet
Total saturates	14.6 ± 0.1^{a,b}	13.8 ± 0.3^{a,b}	13.6 ± 0.8^{a}	14.8 ± 0.8^{b}
Total monoenes	20.9 ± 0.4^{a}	13.2 ± 0.7^{b}	15.4 ± 0.6^{c}	19.2 ± 0.7^{d}
18:2n-6	1.0 ± 0.1^{a}	10.3 ± 0.2^{b}	3.4 ± 0.3^{c}	0.5 ± 0.1^{d}
18:3n-6	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.1
20:2n-6	0.2 ± 0.1^{a}	1.7 ± 0.1^{b}	0.6 ± 0.1^{c}	0.1 ± 0.0^{a}
20:3n-6	0.1 ± 0.0^{a}	1.8 ± 0.3^{b}	0.6 ± 0.1^{c}	0.2 ± 0.1^{a}
20:4n-6	5.6 ± 0.1^{a}	8.3 ± 0.8^{b}	5.3 ± 0.4^{a}	5.4 ± 0.4^{a}
22:5n-6	0.7 ± 0.2	0.6 ± 0.1	0.6 ± 0.3	0.5 ± 0.1
Total n-6	8.2 ± 0.3^{a}	23.4 ± 0.5^{b}	10.8 ± 0.6^{c}	7.3 ± 0.5^{a}
18:3n-3	2.0 ± 0.1^{a}	1.2 ± 0.1^{b}	4.5 ± 0.6^{c}	1.5 ± 0.2^{d}
18:4n-3	trace	trace	0.1 ± 0.0	trace
20:3n-3	—^{a}	—^{a}	0.8 ± 0.1^{b}	0.1 ± 0.0^{c}
20:4n-3	0.3 ± 0.0^{a}	0.1 ± 0.1^{a}	0.7 ± 0.2^{b}	0.3 ± 0.1^{a}
20:5n-3	6.3 ± 0.4^{a}	4.0 ± 0.1^{b}	6.5 ± 0.4^{a}	7.9 ± 0.4^{c}
22:5n-3	1.3 ± 0.1^{a}	0.8 ± 0.1^{b}	1.2 ± 0.2^{a}	1.9 ± 0.1^{c}
22:6n-3	38.4 ± 0.3^{a}	36.0 ± 0.3^{b}	37.3 ± 1.2^{a,b,c}	37.0 ± 0.6^{c}
Total n-3	48.0 ± 0.6^{a}	42.0 ± 0.3^{b}	51.1 ± 2.0^{c}	48.7 ± 0.7^{a}
Total PUFA	56.2 ± 0.8^{a}	65.3 ± 0.4^{b}	61.9 ± 1.5^{c}	55.9 ± 0.9^{a}
n-3/n-6	5.9 ± 0.1^{a}	1.8 ± 0.1^{b}	4.8 ± 0.5^{c}	6.7 ± 0.4^{d}
20:4/20:5	0.9 ± 0.1^{a}	2.1 ± 0.3^{b}	0.9 ± 0.1^{a}	0.7 ± 0.1^{a}
Total DMA	3.9 ± 0.2^{a}	3.9 ± 0.8^{a}	4.5 ± 0.2^{b}	5.5 ± 0.3^{b}

[a]Values are weight % of total fatty acids. Footnotes as described in Table 2; — = not detected; DMA = dimethylacetals. See Table 1 for other abbreviations.

TABLE 4
Fatty Acid Compositions of Phosphatidylinositol from Gill of Salmon Fed Diets Containing Either FO, SO, LO, or MO[a]

Fatty acid	FO diet	SO diet	LO diet	MO diet
Total saturates	36.3 ± 1.0^{a}	39.1 ± 0.2^{b}	38.8 ± 1.4^{b}	39.2 ± 0.4^{b}
Total monoenes	8.7 ± 0.3	6.7 ± 2.5	6.6 ± 1.0	7.6 ± 0.5
18:2n-6	0.4 ± 0.1^{a}	1.6 ± 0.1^{b}	0.5 ± 0.1^{a}	0.1 ± 0.0^{c}
18:3n-6	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.1
20:2n-6	0.2 ± 0.1^{a}	0.5 ± 0.1^{b}	0.3 ± 0.1^{a,b}	trace^{c}
20:3n-6	0.3 ± 0.1^{a}	2.1 ± 0.4^{b}	1.3 ± 0.2^{c}	0.2 ± 0.1^{a}
20:4n-6	30.4 ± 1.0	32.6 ± 3.6	28.4 ± 1.6	30.7 ± 0.8
22:5n-6	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.2 ± 0.1
Total n-6	31.7 ± 0.8^{a}	37.2 ± 3.4^{b}	31.1 ± 1.1^{a}	31.6 ± 0.9^{a}
18:3n-3	0.2 ± 0.0^{a}	0.1 ± 0.0^{a}	0.7 ± 0.2^{b}	0.1 ± 0.0^{a}
18:4n-3	0.2 ± 0.1	trace	0.1 ± 0.0	trace
20:3n-3	—^{a}	—^{a}	0.3 ± 0.1^{b}	0.1 ± 0.0^{c}
20:4n-3	0.2 ± 0.1^{a,b}	trace^{b}	0.3 ± 0.1^{a,c}	0.1 ± 0.0^{b}
20:5n-3	2.6 ± 0.2^{a}	1.2 ± 0.2^{b}	3.5 ± 0.5^{c}	3.7 ± 0.4^{c}
22:5n-3	0.7 ± 0.1^{a,b}	0.5 ± 0.1^{b}	0.8 ± 0.1^{a}	1.3 ± 0.1^{c}
22:6n-3	13.6 ± 0.2^{a}	12.1 ± 1.1^{b}	12.9 ± 0.3^{a,b}	13.7 ± 0.5^{a,b}
Total n-3	17.4 ± 0.3^{a}	13.9 ± 1.2^{b}	18.5 ± 1.0^{a}	19.0 ± 0.3^{a}
Total PUFA	49.2 ± 1.0	51.1 ± 4.3	49.5 ± 1.0	50.6 ± 0.9
n-3/n-6	0.6 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.0
20:4/20:5	12.0 ± 1.3^{a}	28.0 ± 4.0^{b}	8.3 ± 1.4^{c}	8.5 ± 1.0^{c}

[a]Values are weight % of total fatty acids. Footnotes as described in Tables 2 and 3. See Table 1 for abbreviations.

fish fed SO compared to those fed FO. DHA was significantly reduced in fish fed SO compared to fish fed FO. Total n-3 PUFA were significantly lower in fish fed SO compared to the other three treatments. The 20:4/20:5 eicosanoid precursor ratio was significantly higher in fish fed SO compared to all other treatments while the ratio in fish fed FO was significantly higher than in fish fed LO or MO.

The AA and EPA composition and AA/EPA in PC, PE, and PI of salmon kidney are shown in Figures 1–3. In kidney PC (Fig. 1), AA was significantly reduced in fish fed LO compared to the other three treatments. EPA was significantly different for each dietary treatment with the highest value in fish fed MO and the lowest in those fed SO. The AA/EPA ratio was significantly greater in fish fed SO compared to the other three treatments. In kidney PE (Fig. 2), AA was significantly reduced in fish fed LO compared to fish fed FO or SO and was significantly greater in fish fed SO compared to the other three treatments. EPA was sigificantly reduced in SO-fed fish compared to the other three treatments and was significantly increased in fish fed MO compared to the other three groups. As with kidney PC, the AA/EPA ratio was significantly increased in fish fed SO compared to the other three treatments. In kidney PI (Fig. 3) AA was significantly reduced in fish fed LO compared to the other three treatments while AA was significantly lower in fish fed MO compared to those fed SO. EPA was significantly reduced in fish fed SO compared to the other three treatments and was significantly increased in fish fed MO compared to those fed FO. The AA/EPA ratio was significantly greater in fish fed SO compared to the other three treatments and was significantly greater in fish fed FO compared to those fed LO or MO.

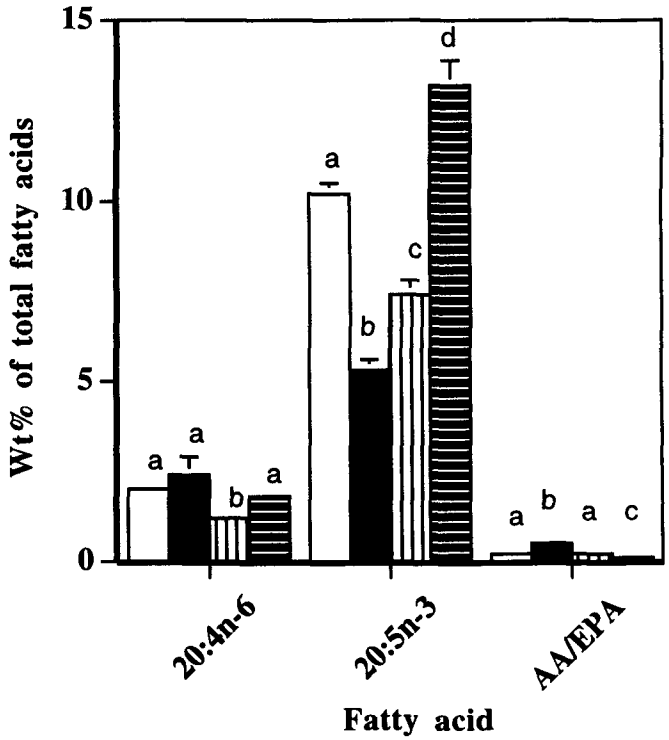


FIG. 1. Levels of 20:4n-6, 20:5n-3, and the ratio of arachidonic acid/eicosapentaenoic acid (AA/EPA) in kidney phosphatidylcholine from salmon fed diets containing either Fosol fish oil (open bars), sunflower oil (filled bars), linseed oil (vertical bars), or Marinol (horizontal bars). Values are means ± SD for four fish per treatment. Values for each fatty acid having a different column letter are significantly different ($P < 0.05$).

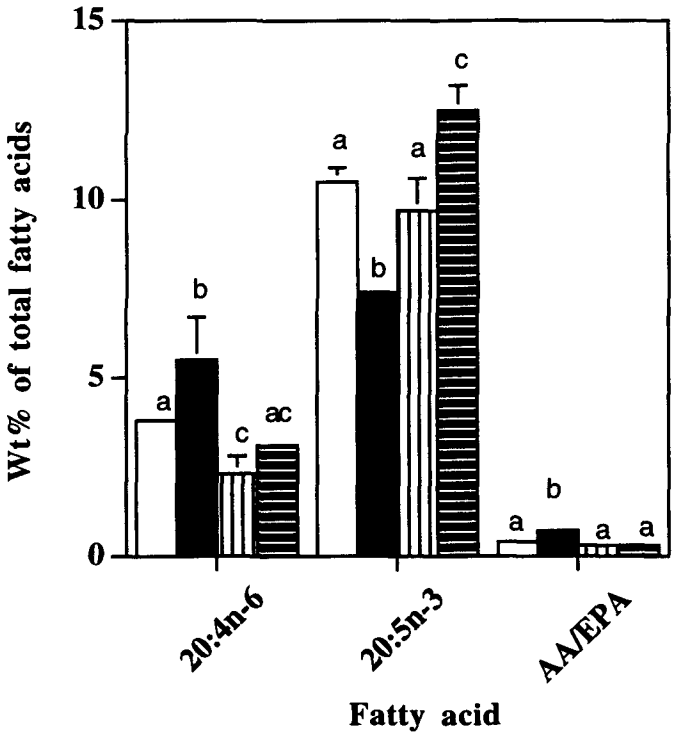


FIG. 2. Levels of 20:4n-6, 20:5n-3, and the ratio of AA/EPA in kidney phosphatidylethanolamine from salmon fed diets containing either Fosol fish oil (open bars), sunflower oil (filled bars), linseed oil (vertical bars), or Marinol (horizontal bars). Values are means ± SD for four fish per treatment. Values for each fatty acid having a different column letter are significantly different ($P < 0.05$).

The activities of gill phospholipase A, measured with endogenous substrate, are shown in Table 5. Results are calculated either as accumulation of free fatty acid or hydrolysis of PC and PE/h per mg protein. In terms of liberation of free fatty acid, the fish fed FO had significantly higher phospholipase A activity compared to the other three treatments with the activity in SO-fed fish being significantly lower than those fed LO. In terms of hydrolysis of PC and PE, the highest mean activity was again in fish fed FO, although this was not significantly different from those fed MO, and the lowest value was in fish fed SO which was significantly lower than in fish fed either FO or MO.

The concentrations of PGE and PGF isomers, of the 2- and 3-series, in gill homogenates are shown in Table 6. $PGF_{3\alpha}$ concentration was significantly greater in gills of fish fed MO compared to the other three treatments. $PGF_{2\alpha}$ concentration was significantly increased in fish fed SO compared to fish fed LO. The ratio of $PGF_{2\alpha}/PGF_{3\alpha}$ was significantly greater

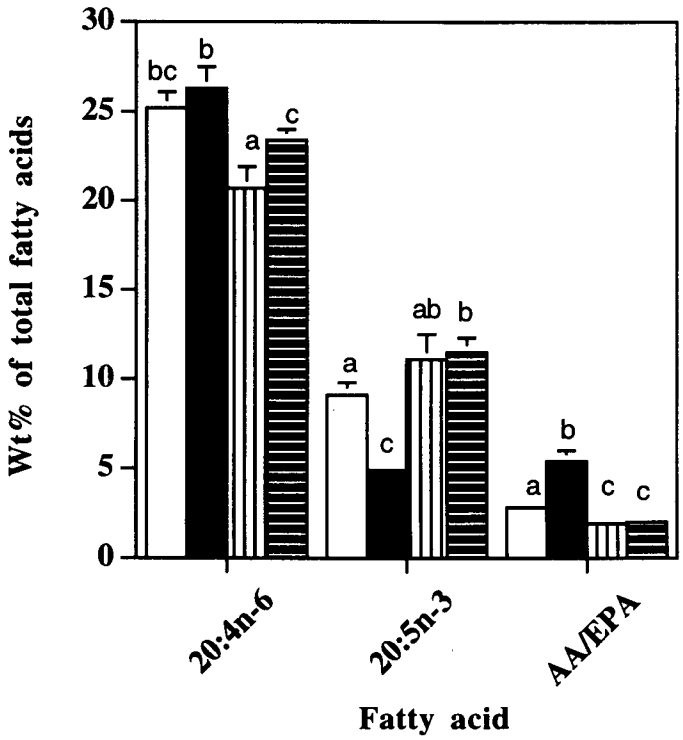


FIG. 3. Levels of 20:4n-6, 20:5n-3, and the ratio of AA/EPA in kidney phosphatidylinositol from salmon fed diets containing either Fosol fish oil (open bars), sunflower oil (filled bars), linseed oil (vertical bars), or Marinol (horizontal bars). Values are means ± SD for four fish per treatment. Values for each fatty acid having a different column letter are significantly different ($P < 0.05$).

in fish fed SO compared to all other treatments. The concentrations of PGE and PGF isomers, of the 2- and 3-series, in kidney homogenates are shown in Table 7. PGE_3 concentration was significantly reduced in kidney of fish fed SO compared to the other three treatments while PGE_2 was significantly increased in fish fed SO compared to those fed FO and LO. Consequently, the PGE_2/PGE_3 ratio was significantly greater in fish fed SO compared to the other three treatments.

TABLE 5
Phospholipase A Activities in Gills of Salmon Fed Diets Containing FO, SO, LO, or MO[a]

Phospholipase A activity	FO diet	SO diet	LO diet	MO diet
nmol fatty acid liberated/hour per mg protein	89.5 ± 10.5[a] (100%)	43.2 ± 5.3[b] (48%)	62.8 ± 3.1[c] (70%)	52.5 ± 4.5[b,c] (59%)
nmol PC and PE hydrolyzed/hour per mg protein	28.9 ± 3.5[a] (100%)	9.8 ± 2.5[b] (34%)	17.2 ± 6.8[b,c] (60%)	22.8 ± 3.7[a,c] (79%)

[a]Values in brackets are percentage of maximum activity. Values are means ± SD for four fish per dietary treatment. Values in the same row with different superscript letters are significantly different ($P < 0.05$); PC, phosphatidylcholine; PE, phosphatidylethanolamine. See Table 1 for other abbreviations.

TABLE 6
Concentrations of PGE_3, PGE_2, $PGF_{3\alpha}$, and $PGF_{2\alpha}$ in Homogenates of Gill from Salmon Fed Diets Containing Either FO, SO, LO, or MO[a]

Prostaglandin	FO diet	SO diet	LO diet	MO diet
PGE_3	11.4 ± 1.3	14.9 ± 2.3	19.4 ± 5.2	16.7 ± 0.5
PGE_2	14.3 ± 1.5	16.1 ± 0.5	11.1 ± 3.9	14.5 ± 4.1
PGE_2/PGE_3	1.3 ± 0.2	1.1 ± 0.3	0.6 ± 0.4	0.9 ± 0.3
$PGF_{3\alpha}$	0.15 ± 0.02[a]	0.10 ± 0.03[a]	0.10 ± 0.01[a]	0.39 ± 0.10[b]
$PGF_{2\alpha}$	0.27 ± 0.09[a,b]	0.44 ± 0.14[a]	0.10 ± 0.02[b]	0.32 ± 0.06[a,b]
$PGF_{2\alpha}/PGF_{3\alpha}$	1.8 ± 0.6[a]	3.5 ± 0.7[b]	1.0 ± 0.2[a]	0.9 ± 0.4[a]

[a]Values are pg/mg tissue and are means ± SD from four samples per dietary treatment. Values in the same row having different superscript letters are significantly different ($P < 0.05$). PGE, prostaglandin E; PGF, prostaglandin F. See Table 1 for other abbreviations.

DISCUSSION

The fatty acid compositions of gill and kidney from fish fed the two fish oils, FO and MO, were largely similar except that fish fed MO incorporated more EPA (up to 42%), and its elongation product 22:5n-3, into membrane phospholipids. This was reflected in a slight reduction in the AA/EPA eicosanoid precursor ratio in most phospholipid classes, although this change was only statistically significant in kidney PC and PI. The concentration of 22:6n-3 was similar or slightly less in phospholipids of fish fed MO compared to those given FO indicating that little of the increased EPA was converted to 22:6n-3 and probably indicated that both diets supplied sufficient 22:6n-3 to satisfy requirements. Fish fed the two plant-derived oils, SO and LO, accumulated increased levels of the principal dietary C_{18} PUFA, 18:2n-6, and 18:2n-6 and 18:3n-3 respectively, into tissue phospholipids. In addition, both treatments had significantly increased levels of 20:2n-6 and 20:3n-6, the products of elongation and of Δ6 desaturation and elongation, in membrane phospholipids compared to fish fed fish oils. However, while fish fed SO had increased levels of AA (the product of Δ5 desaturation) in tissue phospholipids, fish fed LO had AA levels which were significantly less than or equal to the values in fish fed fish oils. It is apparent that LO, which is rich in18:2n-6 but which also contains an abundance of 18:3n-3, has an inhibitory effect on the Δ5 desaturase responsible for conversion of 20:3n-6 to AA.

TABLE 7
Concentrations of PGE_3, PGE_2, $PGF_{3\alpha}$, and $PGF_{2\alpha}$ in Homogenates of Kidney from Salmon Fed Diets Containing Either FO, SO, LO, or MO[a]

Prostaglandin	FO diet	SO diet	LO diet	MO diet
PGE_3	4.1 ± 0.2[a]	2.5 ± 0.4[b]	3.7 ± 0.7[a]	3.8 ± 0.3[a]
PGE_2	2.5 ± 0.2[a]	3.6 ± 0.4[b]	2.6 ± 0.4[a]	2.8 ± 0.4[a,b]
PGE_2/PGE_3	0.6 ± 0.1[a]	1.5 ± 0.1[b]	0.7 ± 0.0[a]	0.7 ± 0.1[a]
$PGF_{3\alpha}$	1.3 ± 0.8	0.8 ± 0.1	2.0 ± 0.9	1.8 ± 0.2
$PGF_{2\alpha}$	1.5 ± 0.2	1.6 ± 0.5	2.5 ± 0.5	1.7 ± 0.3
$PGF_{2\alpha}/PGF_{3\alpha}$	1.5 ± 1.0	2.1 ± 0.5	1.4 ± 0.5	1.0 ± 0.2

[a]Footnotes as described for Table 7. SD <0.05 are tabulated as 0.0. See Tables 1 and 6 for abbreviations.

While the abundance of 18:2n-6 in SO-fed fish allows conversion to AA to proceed unhindered, the presence of competing n-3 PUFA, both 18:3n-3 and C_{20}, in LO-fed fish prevents the same conversion of 18:2n-6 to AA in the latter fish. Thus, in fish fed LO there is competition between 18:2n-6 and 18:3n-3 at the Δ6 desaturase and also between 20:3n-6 and 20:4n-3 at the Δ5 desaturase which results in an overall inhibition of AA production. The ability of diets rich in 18:3n-3 to inhibit conversion of 18:2n-6 to AA has been recorded previously in mammals (9,33), and in fish (20).

The fish fed SO and LO received similar levels of EPA from the diet yet phospholipids from fish fed LO contained significantly more EPA compared to fish fed SO and suggests appreciable conversion of 18:3n-3 to EPA in LO-fed fish. The ability of salmon parr, during the freshwater period of the salmon life cycle, to convert 18:3n-3 to DHA has been recorded previously (13) but as with the fish given pre-formed EPA in the form of MO those given LO did not have increased 22:6n-3 compared to fish fed FO. In general, the reduced EPA and increased AA in SO-fed fish and the increased EPA and reduced AA in LO-fed fish resulted in the former having a significantly higher AA/EPA ratio in tissue phospholipids compared to the latter. In LO-fed fish the EPA formed from 18:3n-3 appeared to be incorporated specifically into PI where the mean EPA values were in excess of those in fish fed FO in both kidney and gill. This further supports previous suggestions that PI may provide a source of precursors for eicosanoid production (34,35).

The fatty acid composition of phospholipids can influence the physical properties of cell membranes which, in turn, can influence the activities of membrane-associated proteins and enzymes (36), including those involved in phospholipid metabolism. Recent studies with rats have shown that an increased dietary n-3/n-6 PUFA ratio, resulting from feeding fish oil, caused increased activity of phospholipase A_2 in gastric mucosa (37) but reduced phospholipase A activity in liver and cardiac myocytes (38,39). In salmon fed a reduced n-3/n-6 PUFA ratio, resulting from feeding SO, the phospholipase A activity was increased in cardiac tissue compared to those fed FO (20). In the present study, fish fed FO had the highest phospholipase A activity followed by those fed MO and LO with the lowest activity in fish fed SO. Clearly the increased incorporation of n-6 PUFA and/or the reduced incorporation of long-chain n-3 PUFA was effective in reducing the activity of phospholipase A in salmon gill. This result explains the reduced production of lipoxygenase metabolites in salmon fed increasing levels of dietary linoleic acid (19). These results demonstrate the importance of phospholipid fatty acid composition in modifying the membrane microenvironment of the phospholipase enzyme which thereby can influence the catalytic activity of the enzyme. The precise mechanism of this process remains unclear, but two hypotheses could be appropriate in this case. Firstly, alterations in membrane PUFA composition could alter the physical characteristics of the membrane, including curvature, viscosity and fluidity, such that association of phospholipase with the membrane phospholipid substrate was altered allowing subsequent changes in phospholipase activity (40). Secondly, phospholipase A_2 is activated by peroxidized fatty acids, and since membranes enriched with long-chain n-3 PUFA have a higher unsaturation index, they may be more susceptible to peroxidation than membranes containing fewer unsaturated bonds (41). In addition to the above influences, PGE_1, derived from 20:3n-6, is known to inhibit release of AA from membrane phospholipids by phospholipases (42). Although PGE_1 concentrations were not measured in the present study, the percentage of 20:3n-6 was increased up to 18-fold, in fish fed SO, and up to 7-fold in fish fed LO, compared to fish fed either of the fish oils.

In addition to their respiratory activity, the gills of euryhaline fish are also involved in osmoregulation. The gills are involved in active transport of Na^+ and Cl^- with the direction of transport determined by external salinity. In euryhaline teleosts, the uptake of Na^+ and Cl^- is activated during adaptation to freshwater to maintain plasma concentrations of the above electrolytes at 120–150 mM (43). In seawater adapted teleosts, the branchial epithelium has an increased population of chloride cells, which are the site of active chloride transport, and also contain most of the Na^+/K^+-ATPase activity found in gill epithelia (44). The teleost kidney in fish adapted to freshwater produces large amounts of very dilute urine while after seawater adaptation, small quantities of very concentrated urine are produced. Kidney water and electrolyte regulation are controlled jointly by hormonal and eicosanoid messengers (15).

Prostaglandins E and F of the 2- and 3-series, derived from AA and EPA, respectively, were measured in homogenates from gill and kidney. Both tissues showed changes in the concentration and ratio of the 2- and 3-series of prostanoids which could be attributed to dietary fatty acid intake. In general, fish fed EPA-rich MO had the highest levels of the 3-series prostanoids, and in some instances these were matched by fish fed LO, while the lowest levels of 3-series prostanoids were in fish fed SO. Apart from gill PGE, the ratio of 2-series/3-series was increased in fish fed SO compared to the other treatments. Thus, the ratio of prostaglandins produced broadly reflected the ratio of precursor PUFA in the tissue phospholipids although comparison of the ratios did not clearly identify one phospholipid as the precursor source.

Although the ability of fish kidney and gill to synthesize prostaglandins has been identified for some time, the precise physiological functions were largely unknown (22,23). In more recent studies the effect of prostaglandins and hydroxy fatty acids, the latter being lipoxygenase metabolites, on osmoregulatory processes have been identified (21,25). Prostaglandins are modulators of fluid and electrolyte balance in fish kidney (45,46) and in gill and opercular membranes (26). PGE_2 and other prostanoids are known to operate in conjunction with prolactin, a hyperosmoregulatory hormone in teleost fish, to control electrolyte balance across gill epithelial membranes (47).

In the present study we have demonstrated that feeding di-

etary lipids with varying amounts of n-3 and n-6 PUFA, derived from fish and vegetable oils, can affect the fatty acid composition, and in particular the incorporation of C_{20} eicosanoid precursors, in salmon gill and kidney. We have also demonstrated the effects of these membrane PUFA alterations on phospholipase A activity and the production of AA- and EPA-derived prostanoids. While mammalian studies suggest that eicosanoids derived from EPA are generally of lower biological potency compared to their AA-derived homologues (48), this may not be true in fish where prostaglandins and leukotrienes derived from EPA and AA appear to have similar functions and efficacy with regard to a number of immunoregulatory activities (49). Clearly, the spectrum of eicosanoids produced by a particular cell is dependent on a more complex mechanistic process than the concentration or ratios of substrate fatty acids esterified in membrane phospholipids alone. The importance of the dietary induced changes described in this study to the physiological functions of osmoregulatory tissues in teleost fish is an area of continuing investigation.

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Effects of Growth Temperature on the Fatty Acid Composition of the Free-Living Nematode *Caenorhabditis elegans*

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ABSTRACT: The effects of growth temperature on the fatty acid compositions of the phosphatidylcholine (PC), phosphatidylethanolamine (PE), and total lipid (TL) fractions of the free-living nematode *Caenorhabditis elegans* were investigated. A reduction in growth temperature from 25 to 15°C caused the proportions of eicosapentaenoic acid (20:5n-3) to increase from 23.6 to 32.5% in the PC, from 7.4 to 10.8% in the PE, and from 12.9 to 19.9% in the TL fractions. Conversely, the levels of dihomo-γ-linolenic acid (20:3n-6) and arachidonic acid (20:4n-6) in these phospholipid fractions and the TL fraction both decreased with decreasing growth temperature. Analysis of the positional distribution of fatty acids in the PC fraction revealed that the change in the composition of C_{20} polyunsaturated fatty acid was obvious in position *sn*-2. Lowering the growth temperature induced an increase in the level of the diacyl subclass of PE from 58% at 25°C to 71% at 15°C, with a concomitant decrease in the levels of the alkylacyl and alkenylacyl subclass of PE of *C. elegans*. These changes observed in the phospholipids of *C. elegans* might be one mechanism for adaptation to low temperature.

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The free-living nematode *Caenorhabditis elegans* has been the subject of a number of recent biochemical studies such as investigations of behavioral genetics and experimental gerontology. Because of its small size and the fact that it is a self-fertilizing hermaphrodite, *C. elegans* can be easily grown under laboratory conditions with *Escherichia coli* as food. Using monoxenic culture conditions, there is no possibility for involvement of intermediate organisms between the nematodes and their food. This offers an advantage in the study of lipid metabolism of this multicellular organism.

Previously, we examined the phospholipid composition and fatty acid compositions of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of *C. elegans* and found that the nematode contained both n-3 and n-6 series polyunsaturated fatty acid (PUFA), and that eicosapentaenoic acid (EPA) (20:5n-3) was the most abundant fatty acid in the PC fraction (1). The abundance of EPA in the total lipids of *C. elegans* was also shown by Hutzell and Krusberg (2). In addition, the free-living nematode species *Turbatrix aceti* and the entomopathogenic nematode *Steinernema carpocapsae* have been shown to contain EPA and to have the ability to synthesize PUFA *de novo* (3–5).

Several lower classes of organisms have been reported to produce EPA *de novo* and accumulate a large amount of EPA to adapt to a decrease in environmental temperature. Shimizu *et al.* (6) showed that several fungal microorganisms produced a large amount of EPA only when grown at low temperature. The increased level of EPA at lower growth temperature was also observed in the *Vibrio* species of bacterium isolated from fish intestine (7) and the nematode *S. carpocapsae* (5).

In the present study, we examined the effects of temperature on the fatty acid compositions and subclass compositions of phospholipids of *C. elegans*. The results showed that the levels of EPA, the most abundant and the most unsaturated fatty acid in *C. elegans*, were increased in the PC, PE, and total lipid (TL) fractions when the nematodes were cultured at low temperature. Furthermore, lowering the growth temperature induced an increase in the level of the diacyl subclass of PE of *C. elegans*.

MATERIALS AND METHODS

Materials. Agar, peptone, tryptone, and yeast extract were obtained from Difco Laboratories (Detroit, MI). Glycerol and precoated thin-layer plates (Silica gel 60) were from Merck (Darmstadt, Germany). Silic AR (100–200 mesh) was the product of Mallinckrodt (Paris, KY). Phospholipase A_2 (EC 3.1.1.4, from *Crotalus adamanteus* venom) was from Sigma Chemical Co. (St. Louis, MO).

Growth of E. coli *and lipid extraction. Escherichia coli* (OP50 strain) was grown in a phosphate buffer (pH 7.4) with tryptone, glycerol, and yeast extract at 37°C. *Escherichia coli* from late-logarithmic cultures was centrifuged at 5,000 × *g* for 10 min. The pelleted *E. coli* was resuspended in S. medium (8) and stored at 4°C until use. For lipid analysis,

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Abbreviations: AA, arachidonic acid; 17:Δ, *cis*-9, 10-methylenehexadecanoic acid; 19:Δ, *cis*-11, 12-methyleneoctadecanoic acid; DGLA, dihomo-γ-linolenic acid; DMA, dimethylacetal; EPA, eicosapentaenoic acid; GC, gas chromatography; NGM, nematode growth medium; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid; TG, triglyceride; TL, total lipid; TLC, thin-layer chromatography.

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E. coli which had been stored at 4°C was homogenized by grinding in a mortar with a pestle in a small amount of chloroform/methanol mixture (1:2, vol/vol). The lipids were extracted from the homogenate according to the method of Bligh and Dyer (9).

Growth of C. elegans. The standard wild-type strain N2 of *C. elegans* was grown at temperatures (±0.5°C) of 15, 20, and 25°C on 90-mm nematode growth medium (NGM) agar plates seeded with *E. coli* as described previously (1). NGM agar plates were prepared as follows: NaCl, 3 g; agar, 17 g; peptone, 5.0 g; cholesterol (5 mg/mL in ethanol), 1 mL and H_2O, 975 mL were mixed, then autoclaved, and 1 M $CaCl_2$, 0.5 mL; 1 M $MgSO_4$, 1 mL and 1 M potassium phosphate (pH 6.0), 25 mL were mixed. The $CaCl_2$, $MgSO_4$, and potassium phosphate solutions were autoclaved separately before addition. Upon observation under the microscope, the worms on plates rich with adult worms were washed off the plates with M9 buffer (8). The suspension of bacteria and nematodes was centrifuged at 170 × *g* for 2 min to pellet the nematodes. The supernatant solution was discarded and the pelleted nematodes were repeatedly washed with M9 buffer. The packed worms were suspended with a small amount of M9 buffer, layered over 35% sucrose solution and centrifuged at 1,800 × *g* for 5 min. *Escherichia coli* and dead worms were sedimented, whereas live *C. elegans* floated on the sucrose layer. The live *C. elegans* was withdrawn and repeatedly washed with M9 buffer to remove sucrose. In another experiment, *C. elegans* was cultured at 25°C until the plates were rich with adult worms on NGM agar plates with *E. coli*. Half of the nematodes were then exposed to 15°C for 5 h before harvesting, and the other half of the nematodes were harvested without low temperature treatment. This shift in temperature was achieved within 30 min.

Lipid extraction and fractionation. Purified live *C. elegans* was homogenized by grinding in a mortar with a pestle in a small amount of chloroform/methanol mixture (1:2, vol/vol). TL were extracted from the homogenate according to the method of Bligh and Dyer (9) as described previously (1). The lipid extracts were fractionated into the neutral lipid fraction and the phospholipid fraction using silic AR column chromatography (1). The PC and PE fractions were prepared from the phospholipid fraction by preparative thin-layer chromatography (TLC) using the solvent system chloroform/methanol/H_2O (65:35:6, by vol). The triglyceride (TG) fraction was prepared from the neutral lipid fraction by TLC using the solvent system petroleum ether/diethyl ether/acetic acid (80:30:1, by vol). Detection was done with 0.01% primuline (in acetone/H_2O, 4:1, vol/vol) under ultraviolet light. The relative amounts of the PC and PE subclass were determined by successive degradation of the phospholipids with mild alkali and acid, respectively (10). Lipid phosphorus was determined by the method of Bartlett (11). The amount of TG was determined by gas chromatography (GC) with arachidic acid (20:0) as the internal standard.

Positional distribution of fatty acid in the PC and PE fraction. PC or PE (1 μmol) of *C. elegans* was dissolved in 3 mL of diethyl ether and 0.4 mL of 25 mM Tris-HCl buffer (pH 6.9) containing 5 mM NaCl and 55 mM $CaCl_2$. The reaction was started by adding 100 units of phospholipase A_2. The reaction mixture was stirred vigorously at room temperature for 2 h. After evaporation of the ether phase under a stream of N_2 gas, 2 mL of a chloroform/methanol mixture (1:1, vol/vol) and 0.5 mL of H_2O were added for phase separation. Lipids were recovered from the chloroform phase and subjected to silic AR column chromatography. Released fatty acids were recovered from the chloroform/methanol mixture (95:5, vol/vol) eluate, and phospholipids were recovered from the methanol eluate. Lysophospholipid was purified from the methanol eluate by preparative TLC. The released fatty acids and fatty acids esterified to lysoPC and lysoPE were subjected to transmethylation, and the resulting fatty acid methyl esters were analyzed by GC as described below. Unhydrolyzed PC and PE were both less than 2% compared to lysoPC and lysoPE, respectively. The hydrolysis of the *sn*-2-esterified fatty acid of PC by phospholipase A_2 was evaluated using 1-palmitoyl-2-[1-^{14}C] linoleoyl-GPC (12).

Fatty acid analysis. Fatty acyl residues of PC, PE, and TG of the *C. elegans* were converted to fatty acid methyl esters with 5% methanolic HCl and analyzed by GC (Shimadzu 14A; Shimadzu, Kyoto, Japan) equipped with a capillary column fused with a 0.25 μm film of polar CBP20 (0.22 mm × 30 m; Shimadzu). The temperature of both the injector and the flame-ionization detector was 250°C. The initial column temperature was set at 170°C and then raised to 225°C at 5°C/min. The fatty acid methyl esters obtained from the total lipid extracts of *C. elegans* were purified before GC by TLC using the solvent system petroleum ether/diethyl ether/acetic acid (80:30:1, by vol) to remove unsaponifiable materials. Fatty acid methyl esters were identified by comparing their retention times with those of authentic standards as described previously (1).

RESULTS

Effects of growth temperature on fatty acid composition of C. elegans. Because the standard wild-type strain N2 of *C. elegans* can be grown at temperatures between 12 and 25°C monoxenically on *E. coli* as a food source (8), we chose temperatures of 15, 20, and 25°C for growth of *C. elegans* in this study. The culture times of nematodes were 9–10 d, 7–8 d, and 6–7 d at 15, 20, and 25°C, respectively. The fatty acid compositions of the PC, PE, and TL fractions of *C. elegans* grown at these temperatures are shown in Table 1. Growth temperature affected the proportions of C_{20} PUFA in the PC, PE, and TL fractions of *C. elegans*. In the PC fraction, the level of EPA was increased from 23.6% at 25°C to 32.5% at 15°C. Conversely, dihomo-γ-linolenic acid (DGLA) (20:3n-6) and arachidonic acid (AA) (20:4n-6) in the PC fraction of *C. elegans* were both decreased with lowered growth temperature. Although the extent of the changes was smaller than that in the PC fraction, the increased level of EPA and the decreased level of AA were also observed in the PE and

TABLE 1
Fatty Acid Compositions (%) of PC, PE, and TL Fractions of *Caenorhabditis elegans* Grown at 15, 20, and 25°C, and Fatty Acid Composition of TL Fraction of *Escherichia coli* Grown at 37°C[a]

	C. elegans									*E. coli*
	PC			PE			TL			TL
Fatty acid	15°C	20°C	25°C	15°C	20°C	25°C	15°C	20°C	25°C	37°C
14:0	0.3 ± 0.1[b]	0.4 ± 0.1	0.5 ± 0.2	0.2 ± 0.1[c]	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0	0.6 ± 0.3	0.5 ± 0.3	4.5 ± 0.5
15:*iso*	0.7 ± 0.1	1.0 ± 0.4	1.0 ± 0.2	0.8 ± 0.3	1.4 ± 0.6	1.4 ± 0.9	1.2 ± 0.1	1.7 ± 0.5	1.6 ± 0.7	N.D.[e]
15:*ante*	0.4 ± 0.2	0.4 ± 0.4	0.4 ± 0.1	0.3 ± 0.1	0.6 ± 0.2	0.8 ± 0.8	0.7 ± 0.1	1.3 ± 0.4	0.9 ± 0.5	N.D.
16:*iso*	0.8 ± 0.1	1.1 ± 0.7	1.0 ± 0.3	0.6 ± 0	0.8 ± 0.5	1.2 ± 0.6	1.0 ± 0.1	1.7 ± 0.4	1.4 ± 0.5	N.D.
16:0	1.4 ± 0.3[b]	2.6 ± 0.8	2.8 ± 0.4	2.8 ± 0.5	3.1 ± 0.5	3.3 ± 0.2	2.0 ± 0.2[c]	2.8 ± 0.9	3.1 ± 0.8	32.5 ± 1.0
16:1n-7	0.9 ± 0.3	1.0 ± 0.3	1.5 ± 0.5	2.2 ± 1.0	2.5 ± 1.1	1.5 ± 0.9	1.4 ± 0.3	1.7 ± 0.4	1.9 ± 0.5	10.8 ± 1.1
17:*iso*	1.8 ± 0.3	2.1 ± 0.5	2.3 ± 0.4	3.5 ± 0.5	2.7 ± 0.7	3.4 ± 0.9	3.0 ± 0.2	3.2 ± 0.3	2.9 ± 1.0	N.D.
17:*ante*	0.5 ± 0.2	0.4 ± 0.1	0.4 ± 0.3	0.9 ± 0.4	0.7 ± 0.3	0.3 ± 0.3	0.7 ± 0.1	1.0 ± 0.4	0.6 ± 0.4	N.D.
17:0	0.6 ± 0.1[b]	0.9 ± 0.2	1.1 ± 0.2	1.2 ± 0.1	1.1 ± 0.2	1.5 ± 0.1	0.9 ± 0.1	1.1 ± 0.3	1.3 ± 0.2	1.3 ± 0.8
17:Δ	3.9 ± 0.5[c]	3.5 ± 1.5	5.2 ± 0.8	3.4 ± 0.3	4.4 ± 2.3	3.3 ± 0.3	6.0 ± 0.9	5.3 ± 1.5	7.1 ± 2.0	22.5 ± 1.3
18:0DMA[d]	0.3 ± 0.1	0.1 ± 0.1	0.3 ± 0.2	12.1 ± 0.3[c]	12.5 ± 3.8	15.0 ± 2.0	4.5 ± 0.2	4.6 ± 1.3	4.8 ± 1.3	N.D.
18:0	5.7 ± 0.5[c]	6.9 ± 2.1	7.2 ± 1.1	7.9 ± 0.5	6.3 ± 0.8	6.7 ± 0.7	6.9 ± 0.3[c]	7.0 ± 1.5	7.4 ± 0.3	0.7 ± 0.1
18:1n-9	2.4 ± 0.6[b]	3.7 ± 0.4	4.4 ± 0.5	1.4 ± 0.2[b]	2.0 ± 0.7	2.4 ± 0.3	2.0 ± 0.2[b]	3.0 ± 1.4	3.5 ± 0.6	N.D.
18:1n-7	11.6 ± 2.2	11.7 ± 1.8	12.0 ± 1.1	20.1 ± 1.5	20.9 ± 1.0	21.4 ± 1.8	14.7 ± 1.7	16.6 ± 1.4	16.3 ± 1.0	11.6 ± 1.9
18:2n-6	8.5 ± 0.9[c]	6.5 ± 1.0	6.3 ± 1.1	13.3 ± 1.3[c]	11.9 ± 3.4	10.7 ± 1.6	10.4 ± 1.0[b]	8.5 ± 1.2	8.0 ± 0.7	N.D.
19:Δ	4.7 ± 0.6[c]	3.5 ± 1.7	3.5 ± 0.6	2.4 ± 0.5[c]	1.8 ± 1.0	1.6 ± 0.5	4.5 ± 0.5	2.6 ± 0.6	3.2 ± 1.1	7.2 ± 0.2
20:3n-6	4.1 ± 0.4[b]	5.8 ± 0.9	6.6 ± 0.9	2.6 ± 0.3	3.8 ± 1.2	3.9 ± 1.7	4.5 ± 0.5[b]	4.8 ± 0.7	6.3 ± 0.6	N.D.
20:4n-6	4.2 ± 0.2[b]	6.3 ± 0.9	7.8 ± 0.8	1.6 ± 0.1[b]	2.3 ± 0.5	2.3 ± 0.3	3.1 ± 0.5[c]	3.7 ± 0.2	4.7 ± 0.8	N.D.
20:4n-3	4.9 ± 0.7	5.7 ± 1.2	4.8 ± 0.7	2.5 ± 0.3	3.6 ± 0.7	3.2 ± 2.4	5.2 ± 0.2	5.0 ± 0.5	5.0 ± 0.4	N.D.
20:5n-3	32.5 ± 0.9[b]	28.5 ± 4.7	23.6 ± 0.7	10.8 ± 0.5[c]	9.8 ± 3.1	7.4 ± 2.0	19.9 ± 1.2[b]	17.4 ± 3.0	12.9 ± 1.5	N.D.

[a]Values are weight percentage, as mean values ± SD (four, five and five harvests of nematodes grown at 15, 20, and 25°C, respectively and three harvests of *E. coli* grown at 37°C). Significantly different from *C. elegans* grown at 25°C as determined using the Student's *t*-test ([b]$P < 0.005$, [c]$P < 0.05$). [d]DMA, dimethylacetal. [e]N.D., Not detected; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TL, total lipid.

TL fractions when the nematodes were cultured at low temperature. The proportions of saturated acids such as palmitic acid (16:0) and stearic acid (18:0) in the PC and TL fractions of the nematodes grown at 15°C were significantly lower than those in the nematodes grown at 25°C. Growth temperature also affected the levels of C_{18} unsaturated fatty acids in *C. elegans*. In the PC, PE, and TL fractions, the levels of oleic acid (18:1n-9) were decreased and the levels of linoleic acid (18:2n-6) were increased with lowering of the growth temperature.

Effects of growth temperature on the positional distribution of fatty acids in the PC and PE fraction of C. elegans. The positional distributions of fatty acids in the PC and PE fraction of *C. elegans* grown at 15 and 25°C were investigated (Table 2). The proportions of C_{20} PUFA located at the *sn*-2 position of PC were higher than those at the *sn*-1 position, but substantial proportions of C_{20} PUFA were present at the *sn*-1 position. The effects of growth temperature on the fatty acid composition of the PC fraction were more obvious in position *sn*-2 than in position *sn*-1. The reduction of growth temperature caused the proportion of EPA at the *sn*-2 position to increase from 34.2 to 42.9%. Although the level of AA esterified at the *sn*-1 position was not significantly affected by growth temperature, its level at the position *sn*-2 was lower at 15°C than that of 25°C (4.9% vs. 8.2%). The proportions of the C_{18} fatty acids and fatty alcohol (detected as dimethylacetal (DMA)) at the *sn*-1 position of PE were markedly influenced by growth temperature. The proportion of 18:0 DMA derived from the predominant alkenyl chain of PE of *C. elegans* (1) was decreased from 20.7% at 25°C to 6.2% at 15°C, in agreement with the results of analysis of PE subclass (see below). Conversely, the levels of stearic acid and *cis*-vaccenic acid (18:1n-7) in position *sn*-1 of PE were both increased with decreasing growth temperature.

Effects of short-term exposure of C. elegans *to low temperature on EPA content in the PC, PE, and TG fractions.* Next, we examined whether short-term exposure of *C. elegans* to low temperature affected the levels of EPA in the PC and PE fractions of the nematodes. In this experiment, we also investigated the change in the content of EPA in the TG fraction to examine the possibility that EPA esterified to TG was transferred to phospholipids as an emergency response to the reduced temperature. The amount of TG was almost equal to that of PC of *C. elegans* (molar ratio) and the proportion of EPA in the TG fraction of nematodes grown at 25°C was around 7%. When the nematodes grown at 25°C for 6–7 d were treated at 15°C for 5 h, the EPA level in the TG fraction was not affected. In contrast, the EPA level in the PC fraction was significantly increased compared to the *C. elegans* maintained at 25°C (Fig. 1). These results indicated that recruitment of EPA from TG to PC would not contribute to the increase in the EPA level in the PC fraction in these experimental conditions.

Effects of growth temperature on subclass composition of PC and PE of C. elegans. As described previously, PE of *C. elegans* contained substantial proportions of the alkylacyl

TABLE 2
Positional Distribution (%) of Fatty Acid in the PC and PE Fraction of *C. elegans* Grown at 15°C and 25°C[a]

	PC				PE			
	15°C		25°C		15°C		25°C	
Fatty acid	*sn*-1	*sn*-2	*sn*-1	*sn*-2	*sn*-1	*sn*-2	*sn*-1	*sn*-2
14:0	2.1 ± 0.8	0.5 ± 0.1	1.1 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.4 ± 0.1[b]	0.9 ± 0.3	0.8 ± 0.1
15:0*iso*	1.0 ± 0.2	0.2 ± 0.1	1.0 ± 0.2	0.4 ± 0.1	2.0 ± 0.5	0.6 ± 0.1[b]	1.8 ± 0.7	1.5 ± 0.5
15:0*ante*	0.6 ± 0.2	0.3 ± 0.1	0.4 ± 0.1	0.1 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.6 ± 0.1
16:0*iso*	2.0 ± 0.6	0.4 ± 0.1	1.5 ± 0.7	0.5 ± 0.1	0.9 ± 0.1	0.8 ± 0.3	0.9 ± 0.3	1.1 ± 0.4
16:0	8.3 ± 3.4	2.3 ± 0.4[b]	8.5 ± 2.9	3.8 ± 0.8	8.7 ± 0.6	1.9 ± 0.4[b]	10.5 ± 2.2	3.3 ± 0.6
16:1n-7	1.4 ± 0.7	0.8 ± 0.1[b]	2.1 ± 1.5	2.2 ± 0.3	1.2 ± 0.2	5.7 ± 1.7	1.6 ± 0.1	5.3 ± 1.1
17:0*iso*	1.8 ± 1.2	0.8 ± 0.2	2.7 ± 0.6	0.9 ± 0.1	7.0 ± 2.7	1.6 ± 0.1	4.8 ± 1.6	1.6 ± 0.3
17:0*ante*	0.2 ± 0.3	0.4 ± 0.2	0.1 ± 0.2	0.5 ± 0.2	0.7 ± 0.1	1.0 ± 0.3	0.9 ± 0.8	1.3 ± 0.6
17:0	1.2 ± 0.2	0.3 ± 0	1.4 ± 0.1	0.4 ± 0.1	3.3 ± 0.3	0.8 ± 0.1	3.4 ± 0.9	0.7 ± 0
17:Δ	5.6 ± 0.8[b]	1.6 ± 0.1[b]	7.7 ± 0.3	2.6 ± 0.5	1.4 ± 0.2	4.6 ± 0.3	8.2 ± 4.4	5.5 ± 1.2
18:0DMA[c]	1.4 ± 1.3	N.D.[d]	0.6 ± 0.3	N.D.	6.2 ± 2.4[b]	N.D.	20.7 ± 4.1	N.D.
18:0	6.5 ± 1.3	2.3 ± 0.4	6.3 ± 1.2	2.4 ± 0.4	22.9 ± 3.1[b]	2.4 ± 0.8	15.5 ± 1.8	2.7 ± 1.3
18:1n-9	7.5 ± 4.3	3.6 ± 0.2[b]	7.3 ± 1.3	5.1 ± 0.9	1.1 ± 0.1	1.7 ± 0.1	1.0 ± 0.6	2.5 ± 0.3
18:1n-7	16.5 ± 2.5	5.9 ± 0.5	17.3 ± 1.0	6.3 ± 1.1	17.7 ± 1.1[b]	22.3 ± 1.0	11.9 ± 1.3	26.1 ± 3.5
18:2n-6	7.1 ± 2.1	9.1 ± 1.8	6.4 ± 0.5	7.8 ± 1.4	2.8 ± 0.7[b]	18.2 ± 0.5	1.4 ± 0.3	15.3 ± 2.9
19:Δ	6.7 ± 2.1	2.2 ± 0.3	7.3 ± 1.2	1.9 ± 0.7	3.7 ± 0.4[b]	2.0 ± 0.5	1.6 ± 0.2	1.7 ± 0.1
20:3n-6	3.1 ± 0.2	5.1 ± 1.0	4.1 ± 0.9	6.3 ± 0.6	1.5 ± 0.1[b]	3.6 ± 0.1	0.9 ± 0.1	4.2 ± 0.4
20:4n-6	2.3 ± 0.7	4.9 ± 0.3[b]	2.6 ± 0.9	8.2 ± 1.7	0.1 ± 0.1	2.3 ± 0.5	0.1 ± 0.2	3.1 ± 1.1
20:4n-3	3.2 ± 0.3	6.9 ± 1.7	2.9 ± 1.0	5.8 ± 0.6	0.7 ± 0.5	4.7 ± 0.6	0.6 ± 0.4	3.8 ± 1.1
20:5n-3	10.9 ± 3.2	42.9 ± 4.5[b]	6.5 ± 2.4	34.2 ± 2.1	0.8 ± 0.4	15.2 ± 1.3	0.5 ± 0.3	13.7 ± 0.7

[a]Values are weight percentage, as mean values ± SD (three harvests of nematodes grown at 15 and 25°C). [b]Significantly different from *C. elegans* grown at 25°C as determined using the Student's *t*-test ($P < 0.05$). [c]DMA, dimethylacetal. [d]N.D., Not detected. See Table 1 for other abbreviations.

and alkenylacyl subclass (1). These subclasses of PE were both decreased with lowered growth temperature. Conversely, the content of the diacyl subclass of PE was increased from 58% at 25°C to 71% at 15°C (Fig. 2). In contrast to the PE fraction, a large portion of PC was accounted for by the diacyl subclass (93.5% at 25°C and 95.3% at 15°C), irrespective of the growth temperature.

DISCUSSION

Under the monoxenic culture conditions used in this study, *E. coli* was the food for *C. elegans*. Despite the fact that the fatty acids which comprised the *E. coli* lipids were saturated, cyclopropane-ringed and n-7 series monoenoic fatty acids (Table 1), *C. elegans* contained n-3 and n-6 series PUFA. This indicates that *C. elegans* has the ability to synthesize these highly unsaturated fatty acids *de novo*, as in the nematodes *T. aceti* (4) and *S. carpocapsae* (5). Although higher animals have been considered unable to synthesize linoleic acid *de novo*, some insect species (13,14), the land slug (15), the garden snail (15), and protozoan amoeba (16), have recently been reported to have ω6 desaturase activity. Both ω6 and ω3 desaturase activities must be present in the multicellular animal *C. elegans*, because the nematodes contain n-6 and n-3 series PUFA. Furthermore, the presence of EPA and AA in *C. elegans* also indicates that this nematode has the ability to desaturate the Δ-6 and Δ-5 positions of fatty acid like higher animals.

Lowering the growth temperature has been shown to induce alterations in membrane fatty acid compositions in various organisms. The nematode *C. elegans* tested here showed an increase in EPA levels and decrease in AA and DGLA levels in the PC and PE fractions when cultured at low temperature. These changes in the composition of C_{20} PUFA in response to a decreased growth temperature agree with similar results in *S. carpocapsae* (5). The increase in the level of EPA and the decrease in the levels of DGLA and AA with lowered growth temperature observed in the PC fraction of *C. elegans* was more obvious in position *sn*-2 than *sn*-1. It appears that the amounts of EPA synthesized *de novo* are increased at lower temperature, because the elevated level of EPA was also observed in the TL fraction of *C. elegans*. A possible explanation of this phenomenon is that the activities of the ω3 and Δ-6 desaturase (both of which deasaturate linoleic acid) were differently affected by the growth temperature. At lower growth temperature, amounts of linoleic acid metabolized by ω3 desaturase might be increased to form more abundant α-linolenic acid. This change might lead to the elevated level of EPA with depressed levels of n-6 series PUFA such as AA. It should be mentioned that an ω3 desaturase activity which produced EPA from AA was present in certain lower fungi (6,17) and the free-living nematode *T. aceti* (4). Further investigations are necessary to elucidate the details of the mechanism underlying the increase in the EPA level in response to a decreased growth temperature in the nematodes.

When the nematodes grown at 25°C for 6–7 d were exposed to 15°C for 5 h, the level of EPA in the PC fraction was significantly increased. In this experiment, the content of EPA in the TG fraction was not affected. Therefore, EPA was rapidly synthesized in response to thermal fluctuations to adapt to low

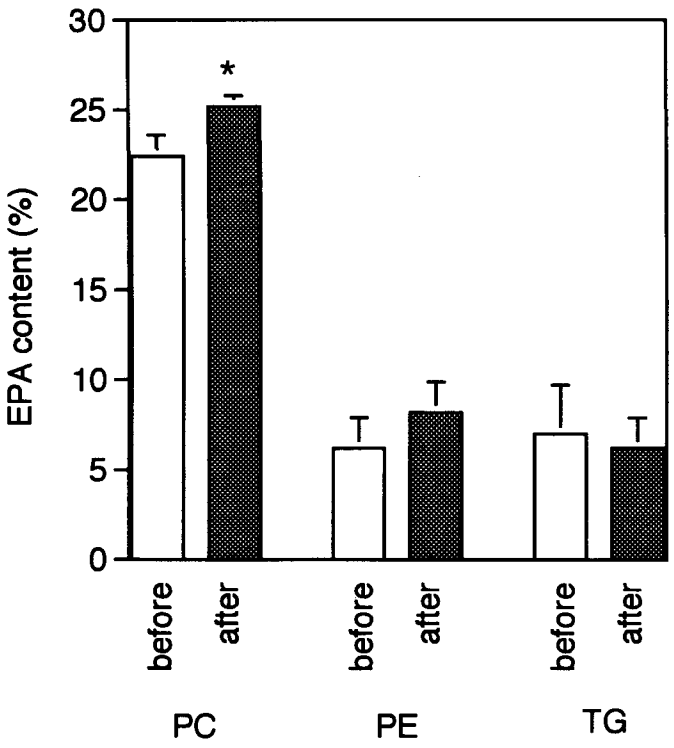


FIG. 1. Effects of short-term exposure of *Caenorhabditis elegans* to low temperature on eicosapentaenoic acid (EPA) content in various lipid classes. Values are weight percent, as mean values ± SD (three harvests of nematodes). *Caenorhabditis elegans* was cultured at 25°C for 6–7 d. Half of the nematodes were then harvested, and the other nematodes were exposed to 15°C for 5 h before harvesting. *Significantly different from the proportion of EPA of *C. elegans* without low temperature treatment as determined using the Student's *t*-test ($P < 0.05$); open bar, before temperature shift; shaded bar, after temperature shift; PE, phosphatidylethanolamine; PC, phosphatidylcholine; TG, triglyceride.

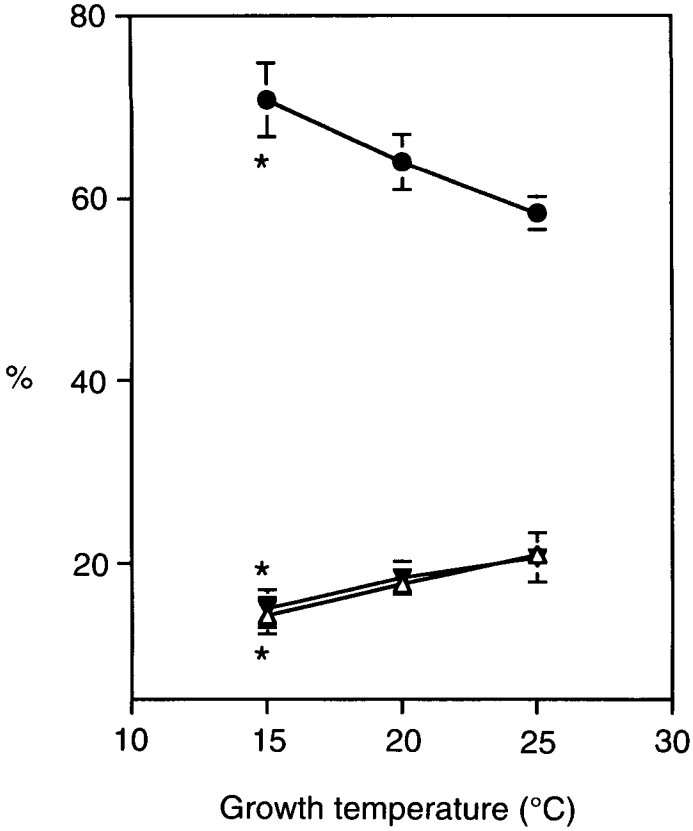


FIG. 2. Effects of growth temperature on the subclass composition of PE of *Caenorhabditis elegans*. *Caenorhabditis elegans* was grown at temperatures of 15, 20, and 25°C. Values are mean ± SD (four, five, and five harvests of nematodes grown at 15, 20, and 25°C, respectively). *Significantly different from the proportion of the corresponding PE subclass of *C. elegans* grown at 25°C as determined using the Student's *t*-test ($P < 0.005$); —●—, diacyl; —△—, alkylacyl; —▼—, alkenylacyl.

temperature rather than through recruitment of EPA from TG to PC.

Growth temperature affected the subclass composition of the PE fraction. At lower growth temperature, the levels of stearic acid and *cis*-vaccenic acid in position *sn*-1 of PE were markedly increased with a concomitant decrease in the level of 18:0 DMA, indicating that the depressed level of alkylacyl and alkenylacyl subclasses of PE were compensated for by the increase in levels of diacylPE containing these C_{18} fatty acids in position *sn*-1. Judging from experiments with monolayers and bilayers, ether bonding has been considered to be less polar than carbonyl ester bonding (18,19). Moreover, PE containing monoenoic acid at position *sn*-1 have been shown to have a disordering effect on biomembranes (20,21). Therefore, the increase in the proportion of PE molecular species with a monoenoic acid in position *sn*-1 might increase the membrane fluidity.

In conclusion, lowering the growth temperature induced the increased level of EPA in the PC, PE, and TL fractions of *C. elegans*. The proportion of the diacyl subclass of PE was also increased with lowered growth temperature. Such changes observed in the phospholipids of *C. elegans* might be one mechanism for the adaptation to low temperature.

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Sterol and Steryl Ester Regulation of Phospholipase A_2 from the Mosquito Parasite *Lagenidium giganteum*

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ABSTRACT: *Lagenidium giganteum,* a facultative parasite of mosquito larvae, cannot synthesize sterols, and requires an exogenous source of these lipids in order to enter its reproductive cycle. This parasite grows vegetatively in the absence of sterols, but requires cholesterol or structurally related compounds to produce motile zoospores, which are the only stage capable of infecting mosquitoes. Sterols structurally related to cholesterol and some steryl esters inhibited the activity of *L. giganteum* phospholipase A_2 (PLA_2), an enzyme that hydrolyzes fatty acids from the *sn*-2 position of glycerophospholipids. Sterols that induce reproduction inhibited *L. giganteum* PLA_2 activity, while sterols and steroids that do not support sporulation had minimal effect. Most steryl esters had no effect on enzyme activity, but cholesteryl arachidonate (CA) was a potent inhibitor of parasite PLA_2. Not all enzymes partly purified using a DEAE-Sephacel column were affected by these lipids, demonstrating selective inhibition of specific enzymes. Potency was enhanced by up to several orders of magnitude if epoxy fatty acids were esterified to the cholesterol nucleus. The steryl ester pool was dynamic during morphogenesis, and the fatty acid composition of the steryl esters did not mimic total cell or membrane (glycerophospholipid) fatty acid composition as *L. giganteum* proceeded through its growth cycle. Synthesis of CA and monoepoxy CA by the parasite was confirmed using electrospray mass spectrometry and collision-induced dissociation. Steryl derivatives selectively inhibited PLA_2 enzymes from bovine pancreas, snake venom, and human cytoplasmic 85-kDa PLA_2.
Lipids 31, 1179–1188 (1996).

Three decades ago it was discovered that sterol auxotrophic Oomycetes found in the genera *Pythium* and *Phytophthora*, which include primarily plant parasites, require an exogenous source of sterols in order to complete their sexual cycle (1–3). It was subsequently found that a third member of this group, *Lagenidium giganteum* (Oomycetes: Lagenidiales), a facultative parasite of mosquito larvae, also requires exogenous sterols structurally related to cholesterol to enter its asexual (4) and sexual (5) cycles. These Oomycetes, a group classically regarded as fungi but now recognized as being allied with diatoms and other chromist algae (6,7), grow vegetatively in the absence of sterols. Although the subject of numerous investigations over the last 30 years (reviewed in Refs. 8–10), the physiological bases of sterol-mediated morphogenesis of these Oomycetes are not known. Most investigations have focused on structure–activity studies reiterating the dual structural and regulatory roles of sterols that had previously been documented in other organisms (11–13).

Oosporogenesis, the production of sexual propagules, can be initiated in the absence of sterols in species of *Phytophthora* and *Pythium* (but not by *L. giganteum*) especially when growth media are supplemented with glycerophospholipids containing unsaturated fatty acid moieties (14). Viable oospores, however, could not be produced without minimal levels of sterol supplementation (15). The most interesting finding regarding sterol-mediated morphogenesis of these auxotrophs was the pivotal role of the ring and side-chain structural features during the onset of meiosis (16). Abortion of oospores not supplemented with cholesterol occurred during the early stages of meiosis, usually soon after fusion of male and female gametangia.

Phospholipases A_2 (PLA_2) are a family of enzymes that hydrolyze fatty acids in the *sn*-2 position of glycerophospholipids, resulting in release of a free fatty acid and a lysophospholipid (17,18). These widespread enzymes have a variety of physiological roles including digestion, membrane synthesis and remodeling, as major components of snake and bee venom, and in providing free arachidonic acid (20:4n-6) for subsequent metabolism by lipoxygenase, cyclooxygenase, and other fatty acid oxygenase enzymes. Oxygenated fatty acids, especially arachidonic acid derivatives collectively known as eicosanoids, are lipid mediators of many inflammatory and allergic reactions. The search for selective inhibitors of PLA_2, therefore, is the focus for much research in both academic and pharmaceutical laboratories (19–21).

Recently we showed that log growth phase cultures of *L. giganteum* growing on defined media supplemented with

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Abbreviations: CAE, cholesteryl arachidonate epoxides; ESI–MS, electrospray–mass spectrometry; ESI–MS/MS, electrospray tandem mass spectrometry (collision-induced dissociation); FAME, fatty acid methyl esters; GC, gas chromatography; GPL, glycerophospholipids; PLA_2, phospholipase A_2; cholesteryl-9-HODE, cholesteryl-9-hydroxy-10,12-octadecadienoic acid; cholesteryl-13-HODE, cholesteryl-13-hydroxy-9,11-octadecadienoic acid; SE, steryl esters; TLC, thin-layer chromatography.

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cholesterol had over four times as much PLA_2 activity as comparable cultures grown in the absence of sterol (22), possibly due to increased rates of membrane synthesis or remodeling. This difference disappeared when enzyme activities in stationary phase cultures were compared. Subsequent *in vitro* assays demonstrated that cholesterol and related sterols added to cell homogenates directly affected PLA_2 activity. Assays for screening PLA_2 inhibitors are fraught with difficulty because of interfacial activation (enzyme activity for micellar and other aggregates can be up to three orders of magnitude greater than that for monomeric substrates) and related complications associated with the composition, concentration, and availability of substrate (23–25). Many PLA_2 inhibitors added at millimolar or high micromolar concentrations act simply by denying the enzyme access to the substrate rather than by specifically interacting with the enzyme (26). Taking these potential complications into consideration, this report extends our initial observations to the effect of steryl esters and hydroxy and epoxy derivatives of steryl esters on enzyme activity. Selective inhibition of PLA_2 from different organisms by various compounds belonging to this new class of inhibitor, including a human enzyme implicated in inflammation, is demonstrated.

MATERIALS AND METHODS

Materials. Enzymes, cold substrate, and other chemicals were obtained from Sigma (St. Louis, MO) or Aldrich Chemical Company (Milwaukee, WI) unless otherwise noted. The cholesteryl esters of cholesteryl-9-hydroxy-10,12-octadecadienoic acid (9-HODE) and cholestery-13-hydroxy-9,11-octadecadienoic acid (13-HODE) were from Cayman Chemical (Ann Arbor, MI). Radiolabelled glycerophospholipids were from New England Nuclear (Boston, MA).

Source and maintenance of the parasite. Cultures of the California isolate of *L. giganteum* (ATCC 52675) were maintained on two types of media. Defined medium designated DM2 (43) consisted of (gram/liter distilled water): 4 glucose, 2 glutamic acid monosodium salt, 1 asparagine, 0.036 $MnCl_2 \cdot 4\ H_2O$, 0.01 $ZnSO_4 \cdot 7H_2O$, 0.063 H_3BO_3, 0.008 $CuSO_4 \cdot 5\ H_2O$, 0.01$(NH_4)_6Mo7O_2 \cdot 4\ H_2O$), 0.017 $CoCl_2 \cdot 6\ H_2O$, 0.012 $Fe(NO_3)_3 \cdot 9\ H_2O$, 0.05 EDTA, 0.05 KH_2PO_4, 0.05 K_2HPO_4, 0.1 thiamine HCl, 0.1 KNO_3, 0.05 methionine, 0.1 $MgSO_4 \cdot 7\ H_2O$), 0.15 $CaCl_2 \cdot 2\ H_2O$, pH 7.0. Reproductively competent cultures were grown on PB2 medium (gram/liter distilled water): 2 Proflo cottonseed extract (Traders Protein, Memphis, TN), 1.5 glucose, 0.5 lactalbumin hydrolysate, 1.0 egg yolk, 0.225 $CaCl_2 \cdot 2\ H_2O$, 0.3 $MgCl_2 \cdot 6\ H_2O$, 0.008 $Fe(NO_3)_3 \cdot 9\ H_2O$, 0.1 fish peptone, pH 7.5. DM2 cultures were grown in 1 L of media in 2800 mL Fernbach flasks (Scientific Products, McGaw Park, IL), and PB2 cultures were maintained in 200 mL of media in 1 L flasks, on a gyrotary shaker for two to seven days.

Homogenization and column chromatographic separation of phospholipase fractions. Cells harvested by filtration on Whatman filter paper (Maidstone, England) were homogenized in lysing buffer (50 mM Tricine, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride dissolved in ethanol, pH 8.5) in a stainless-steel cup with a Virtis (Lab-Line Instruments, Melrose Park, IL) homogenizer for 6–8 min, with liquid nitrogen added approximately every 2 min. Cell disruption was confirmed microscopically. The mycelial homogenate was centrifuged at 10 K × *g* for 30 min and stored at –20°C until use.

Fractions containing PLA_2 activity were obtained by homogenate separation on a 23 × 120-mm DEAE-Sephacel column (Pharmacia) using a NaCl salt step gradient (27). Homogenate containing approximately 15 mg protein (Bio-Rad protein assay; Richmond, CA) was layered on the column and fractionated with eluting buffer (10 mM KCl, 1 mM DTT, 100 mM Tris-HCl, pH 8.0) with 0, 100, 500, and 800 mM NaCl (100 mL of each). Ten mL aliquots were collected, and these were then stored in 1–1.5 mL aliquots at –70°C until use. Fractions were screened for PLA_2 activity with and without cholesterol. Selected fractions exhibiting sterol-mediated enzyme activity were used in subsequent assays.

PLA_2 assay. Radiometric PLA_2 assays were performed using L-α-1-*O*-hexadecyl-2-[5,6,8,9,11,12,14,15-^{3}H]-arachidonoyl phosphatidylcholine (*ca.* 55 mCi/mmol; New England Nuclear) as substrate, following a slight modification of methods developed by Gronich *et al.* (28) and Ghomaschi *et al.* (29). For all enzymes except for the human 85 kDa cytoplasmic PLA_2, the reaction mixture consisted of 4 μL of 30 mM $CaCl_2 \cdot 2\ H_2O$, 3 μL substrate dissolved in DMSO (final substrate concentration of 9.9 μM), and 34 μL enzyme solution. For the 85 kDa enzyme, the buffer in which the enzyme was shipped was driven off by nitrogen, and the enzyme resuspended in 0.5 mL of 80 mM glycine containing 200 μM Triton X-100 (Sigma), 5 mM $CaCl_2 \cdot 2\ H_2O$, 250 μg fatty acid-free bovine serum albumin mL^{-1}, 70% (vol/vol) glycerol, pH 9.0 (30). The reactions were carried out in 1.5 mL glass vials in a shaking water bath for 30 min at 30°C. Enzyme concentrations were adjusted so that in control assays approximately 40–50% of the substrate was metabolized. (The theoretical maximum is approximately 50% substrate hydrolysis.) Stock solutions of steroids, sterols, and steryl esters in hexane were dried under N_2 in reaction vials, and their effects on PLA_2 activity were assessed following 15-min preincubation with shaking at 30°C in the absence of substrate. The reaction was stopped with 370 μL of $CHCl_3/CH_3OH/HCl$ (200:100:1, by vol), followed by sequential addition of 460 μL of $CHCl_3/CH_3OH/H_2O$ (1:2:0.8, by vol), 240 μL $CHCl_3$, 240 μL H_2O, and 10 μL of a carrier solution (stock solution consisting of 400 μL arachidonic acid plus 600 μL of 50 μg/mL 2,6-di-*tert*-butyl-4-methylphenol in DMSO). After vortexing, the top layer was removed, and the bottom layer collected. The concentrated bottom layer was applied to a silica gel column (5–6 cm) in a Pasteur pipet. Free fatty acids were eluted with *ca.* 3.5 mL hexane/diethyl ether/HOAc (70:30:1, by vol) into 4 mL Wheaton Omnivials. The eluent was evaporated under N_2 and suspended in aquasol from New England Nuclear (Wilmington, DE) for liquid scintillation counting analysis.

Data are reported as mean ± 1 SD ($n \geq 3$), and have been normalized to controls.

Synthesis and thin-layer chromatography (TLC) of epoxy fatty acid steryl esters (SE). Epoxide derivatives of cholesteryl arachidonate, cholesteryl linoleate, and cholesteryl linolenate were synthesized using the method of Falck *et al.* (31). Ten mg of the SE was dissolved in 2.61 mL ice cold CH_2Cl_2 and 6.33 mg of 3-chloroperoxybenzoic acid slowly added. Following incubation for 12–15 h at 0°C, 16.67 µL of methyl sulfide was added and the mixture held for 20 min at room temperature. After dilution with 5 mL CH_2Cl_2, the epoxy derivatives were washed in a separatory funnel three times with 5 mL H_2O and then once with 5 mL NaCl-saturated H_2O. The bottom (organic) layer was dried under N_2, the residue resuspended in $CHCl_3/H_2O$ (1:1, vol/vol), the H_2O layer discarded, and the organic layer dried. The epoxides were weighed and stored in MeOH at −70°C. A portion of the mixture was separated on silica gel G plates using hexane/diethyl ether/acetic acid (70:30:1, by vol). A prescored portion of the plate was broken off and the epoxides visualized by exposure to I_2 vapor. Individual bands were eluted from the plate with MeOH and the fractions stored in the same solvent at −70°C. Epoxides were hydrolyzed into polyols by suspension in tetrahydrofuran/H_2O/perchloric acid (18:8.5:0.175, by vol) and stirred for 8 h at room temperature. The reaction was terminated by adding 20 mL H_2O followed by extraction with CH_2Cl_2 (32). The organic layer was dried and the derivatives stored at −70°C until use.

Whole cell, SE, and glycerophospholipid fatty acid analysis. Cultures of the parasite were grown in liquid shake culture on PB2 medium, and separate flasks harvested during log growth phase (15-h post-inoculation), at the time of initiation of oosporogenesis (35-h post-inoculation), and during stationary growth (4-d post-inoculation). Following collection by filtration, total cell lipids were extracted by standard Folch extraction (33). Glycerophospholipids were eluted just above the origin, and the SE fraction collected from near the solvent front following TLC using the system described for the SE epoxides. Fatty acid methyl esters (FAME) were obtained using acetyl chloride following the one-step transesterification procedure of Lepage and Roy (34). FAME were analyzed on a Hewlett-Packard 5890 Series II gas chromatograph (Palo Alto, CA) using a 30 m DB-23 column (J&W Scientific, Folsom, CA). The initial temperature of 120°C was held for 2 min then raised at 4°C per min up to 240°C and held for 10 min. Using He as a carrier gas at 2 mL/min and the constant flow mode of gas chromatography (GC) operation, splitless sample injection was at 200°C and the detector held at 280°C. FAME were identified by comparison with retention times of standards. Fatty acid composition for *L. giganteum* grown in PB2 had previously been confirmed by GC–mass spectrometry (MS) and electrospray (ESI)–MS.

ESI–MS. ESI–MS and tandem mass spectrometry (collision-induced dissociation, ESI–MS/MS) was performed using a triple quadrupole Sciex API III instrument (Thornhill, Ontario, Canada). Samples were dissolved in 5 mM ammonium acetate in methanol, and infused into the electrospray source *via* a 50 µM silica transfer line using a Harvard Apparatus pump at 2–3 µL/min. Positive ion ESI–MS and ESI–MS/MS used an orifice voltage of 65–75 V and an interface temperature of 52°C. For ESI–MS/MS precursor ions were selected with the first of three quadrupoles (Q1) for collision-induced dissociation with argon in the second quadrupole (Q2). The third quadrupole (Q3) was scanned with a mass step of 0.20 Da and 1 ms per step. Precursor ion transmission and related parameters were adjusted so as to achieve slightly better than unit resolution (35). Spectra were collected and analyzed using proprietary software from Sciex.

RESULTS

Enzyme stability and endogenous inhibition. Lagenidium giganteum PLA_2 activity in 10 K × *g* supernatant was stable for several months at −20°C; however, after separation on the DEAE-sephacel column, enzyme activity was rapidly lost if the fractions were stored at less than −70°C. Individual column fractions lost activity if they were thawed more than 2–3 times. Potent endogenous PLA_2 inhibitors were removed during the column chromatographic separation. These compounds could be extracted from 10 K × *g* supernatant with methylene chloride, and two inhibitory fractions were obtained following TLC—one that comigrated with phospholipid standards and a second with an R_f value similar to SE. These inhibitors were not further characterized.

Sterol and steroid regulation of PLA_2 activity. At a concentration of 600 µM, sterols that induce *L. giganteum* oosporogenesis, including ergosterol, 7-dehydrocholesterol, 5-cholestene 3β, 4β-diol, and cholesterol, inhibited PLA_2 activity of crude 10 K homogenates by 55–65% (Fig. 1). Cholestane, desmosterol, cholesterol-5α,6α-epoxide, and ecdysteroids (insect molting hormones), which do not promote reproduction, were found to have minimal effect. Sterols that have some biological activity but that cannot induce appreciable numbers of viable oospores, such as lanosterol and epicholesterol, were found to have an intermediate effect (*ca.* 40–50% inhibition). There was not a well-defined correlation between oospore induction and PLA_2 inhibition in all cases. Ergosterol, which is very effective in inducing *L. giganteum* sporulation if care is taken to minimize degradation of the conjugated ring system during medium preparation, was not significantly more effective than compounds such as epicholesterol and lanosterol that induce minimal reproduction (Fig. 1). There was some variability in the relative effect of sterols on PLA_2 activity in homogenates from different *L. giganteum* cultures, probably due to small differences in the production and activation of at least eight different enzymes capable of hydrolyzing fatty acyl esters in the *sn*-2 position (43), some of which were unaffected by sterols. Cholesterol at the same concentration (600 µM) did not have a significant effect on the activity of two commercially available enzymes, bovine pancreas and *Naja naja* venom PLA_2 (Sigma).

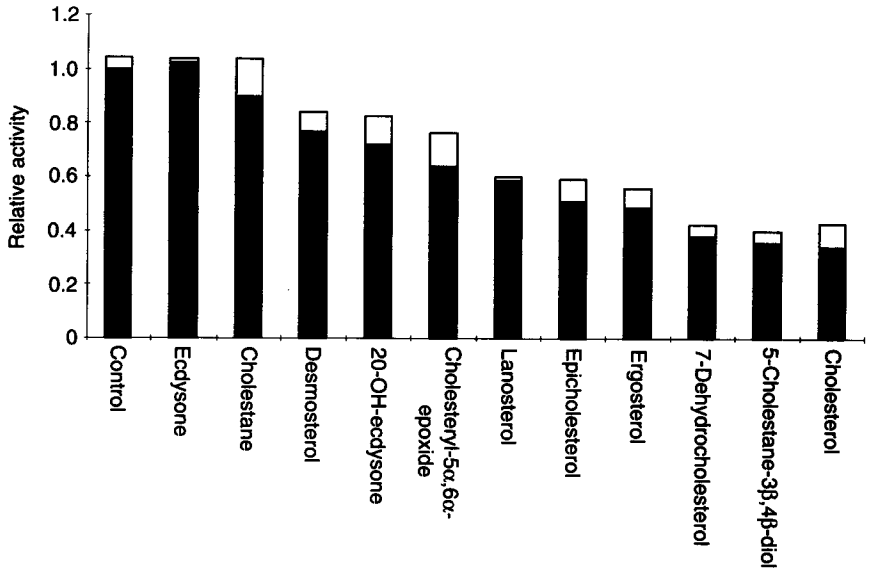


FIG. 1. Effect of sterols (600 μM) on phospholipase A_2 (PLA_2) activity of 10 K × *g* homogenates from *Lagenidium giganteum*. Data is expressed relative to control activity (mean ± SD, $n \geq 3$).

SE. SE were found to have variable effects. Tested on two active *L. giganteum* fractions from a DEAE-Sephacel column that were inhibited by cholesterol (100 mM NaCl/fraction 5 and 800 mM NaCl/fraction 2), cholesteryl palmitate and cholesteryl linoleate, and a number of short-chain esters were not inhibitory at 600 μM (Fig. 2), but cholesteryl arachidonate inhibited PLA_2 activity by 88% at the same concentration.

Cholesteryl-9-HODE and cholesteryl-13-HODE were found to have variable effects on different enzymes. When tested against *L. giganteum* enzymes at 600 μM, the cholesteryl ester of 9-HODE inhibited substrate hydrolysis by about 53%, while the 13-HODE derivative gave no significant inhi-

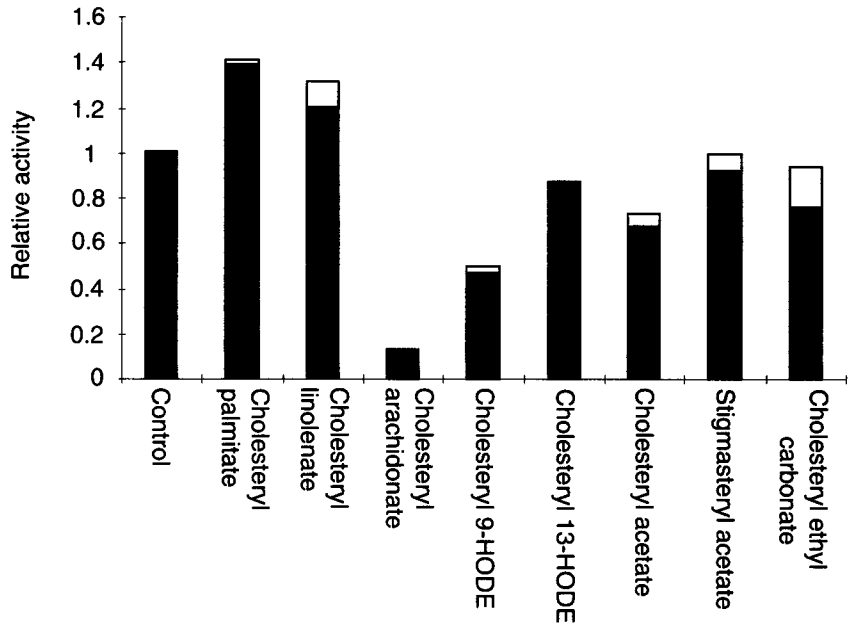


FIG. 2. Effect of steryl esters (600 μM) on PLA_2 activity of 10 K × *g* homogenates from *Lagenidium giganteum*. Data is expressed relative to control activity (mean ± SD, $n \geq 3$). See Figure 1 for abbreviation.

bition (Fig. 2). When tested at the same concentration against the PLA_2 from *Naja naja* venom, both the 9-HODE and 13-HODE cholesteryl esters were found to be extremely inhibitory, with 90 and 92% inhibition, respectively. Human recombinant 85 kDa cytoplasmic PLA_2 was inhibited by over 75% by cholesteryl-13-HODE.

Except for a role in lipid transport, SE are largely considered to be metabolically inert storage compounds. Following separation of SE and glycerophospholipid (GPL) fractions from other lipids using TLC, cycling of the fatty acyl constituents during *L. giganteum* morphogenesis was followed by GC of the FAME. For some fatty acids, e.g., palmitic acid (16:0), there were minor changes in the mol% fatty acyl composition of the SE fraction as the parasite proceeded from log growth, to the initiation of sexual reproduction, and then to stationary phase growth (Table 1). There were significant changes in the mol% fatty acyl composition of other species, especially involving oleic (18:1n-9) and linoleic (18:2n-6) acids (Table 1). Whole cell and GPL fatty acid composition (relative mol%) also changed during morphogenesis, but did not necessarily match the SE fatty acyl composition. This was especially true for arachidonic acid (20:4n-6) where there were very great increases in this polyunsaturated fatty acid during the transition from log growth to later stages in morphogenesis in the whole cell and GPL fractions that was not mirrored by increases in the SE fraction (Table 1).

Positive ion ESI–MS and ESI–MS/MS were used to analyze the SE fraction from a stationary phase culture of the parasite. It was determined that *L. giganteum* synthesized cholesteryl arachidonate and the monoepoxy derivative of this ester (see spectra below). It was not possible to determine the specific epoxy isomer synthesized by the parasite using this spectrometric method.

TABLE 1
Mol% Fatty Acid Composition of Cell, GPL, and SE from Three Stages in the Life Cycle of *Lagenidium giganteum*[a]

	Mol% composition[b]								
	16 h			35 h			4 d		
Fatty acid	Cell	GPL	SE	Cell	GPL	SE	Cell	GPL	SE
14:0	0.5	1	3.6	1.4	1.1	6.1	0.9	1.6	4.3
16:0	21.8	17.1	14.1	33.7	21.6	21.8	31.8	25.5	19.4
16:1	2.9	1.1	7.5	2.9	0.6	4.3	2.2	0.7	3.1
18:0	14.0	41.0	29.7	30.0	16.1	37.0	21.0	15.6	19.4
18:1	14.8	1.2	28.8	1.9	0.5	2.9	1.1	0.6	2.1
18:2	21.3	1.1	4.8	1.2	1.0	18.0	0.8	0.9	35.7
α-18:3	12.8	3.6	trace	1.1	0.8	trace	0.7	0.9	trace
20:0	1.7	1.5	trace	1.7	2.1	trace	1.5	2.7	trace
20:4	9.6	30.5	10.6	24.0	55.1	8.9	39.5	50.0	11.0
20:5	0.3	0.9	0.6	trace	0.7	0.7	0.4	1.0	4.7
22:6	0.3	1.0	trace	2.0	0.4	trace	trace	0.5	trace

[a]16 h: log growth phase; 35 h: initiation of oosporogenesis; 4 d: stationary growth phase.
[b]Trace—<0.1 mol%. There were at least a dozen other minor fatty acids present. Cell, whole cell fatty acid methyl esters (FAME) extract; GPL, FAME of glycerophospholipids; SE, FAME of steryl esters.

Epoxy SE. Of all compounds tested, the mix of cholesteryl arachidonate epoxides (CAE) was the most potent *L. giganteum* PLA_2 inhibitor, with over 90% inhibition of 10 K × *g* supernatant enzyme activity at 630 μM, and inhibition of over 80% of some of the partly purified *L. giganteum* enzymes at concentrations as low as 6 nM. Note that here and in following sections where we are dealing with mixtures of epoxide or polyol isomers, the approximate mid-range molecular weight of the mixtures, e.g., that corresponding to the diepoxy linolenate derivative, was used to generate approximate inhibitor molarities. ESI–MS and ESI–MS/MS examination of the CAE revealed a mixture of mono-, di-, tri-, tetra, and pentaepoxide isomers (Fig. 3).

A preliminary separation of the mixture was done using normal-phase TLC plates with a mobile phase of hexane/ether/glacial acetic acid (70:30:1, by vol). ESI–MS/MS analysis of the TLC fractions revealed that the tetra- and pentaepoxides remained near the origin, with mono- and diepoxides migrating close to the solvent front. When tested for their effects on the activity of an active fraction eluted from a DEAE-sephacel column (100 mM NaCl/fraction 5), most of the epoxide isomers were found to be inhibitory. TLC fractions 1 and 2 (monoepoxides), 5–7 (triepoxides), and 9 and 10 (tetra- and pentaepoxides) were found to be significantly inhibitory at 60 μM (Table 2). When added at 6 μM, the effect on PLA_2 activity of some of the epoxide TLC fractions was reversed, with activity increasing. Lower concentrations of the different fractions were not evaluated.

The activity of human recombinant 85 kDa PLA_2 was significantly increased by preincubation with the CAE mix from concentrations of 6 nM to 60 μM. Because of the inhibition found with cholesteryl-13-HODE (18-carbon hydroxy fatty acid moiety), we synthesized epoxy derivatives of cholesteryl linoleate (18:2n-6 fatty acid) and cholesteryl linolenate (18:3n-3). Both mixtures of epoxide isomers inhibited the 85 kDa enzyme at a concentration of 50 μM, but had no effect on *L. giganteum* PLA_2. The mixture of epoxylinolenate de-

TABLE 2
Effect of Cholesteryl Arachidonate Epoxides on *Lagenidium giganteum* Phospholipase A_2 Activity[a]

TLC fraction (R_f value)	Number of epoxy groups	Relative activity	
		60 μM	6 μM
0.11	4, 5	0.13 ± 0.03	1.11 ± 0.13
0.26	4	0.10 ± 0.02	0.91 ± 0.09
0.34	3	0.69 ± 0.11	1.33 ± 0.09
0.40	3	0.17 ± 0.01	1.51 ± 0.06
0.44	3	0.13 ± 0.02	1.92 ± 0.12
0.49	3	0.18 ± 0.10	1.39 ± 0.05
0.54	3	0.81 ± 0.08	0.87 ± 0.08
0.61	2	0.70 ± 0.22	1.08 ± 0.10
0.71	1, 2	0.12 ± 0.01	1.06 ± 0.05
0.83	1	0.10 ± 0.01	1.22 ± 0.18

[a]The number of epoxy groups in each thin-layer chromatography (TLC) fraction was determined using positive ion electrospray tandem mass spectrometry (ESI–MS/MS).

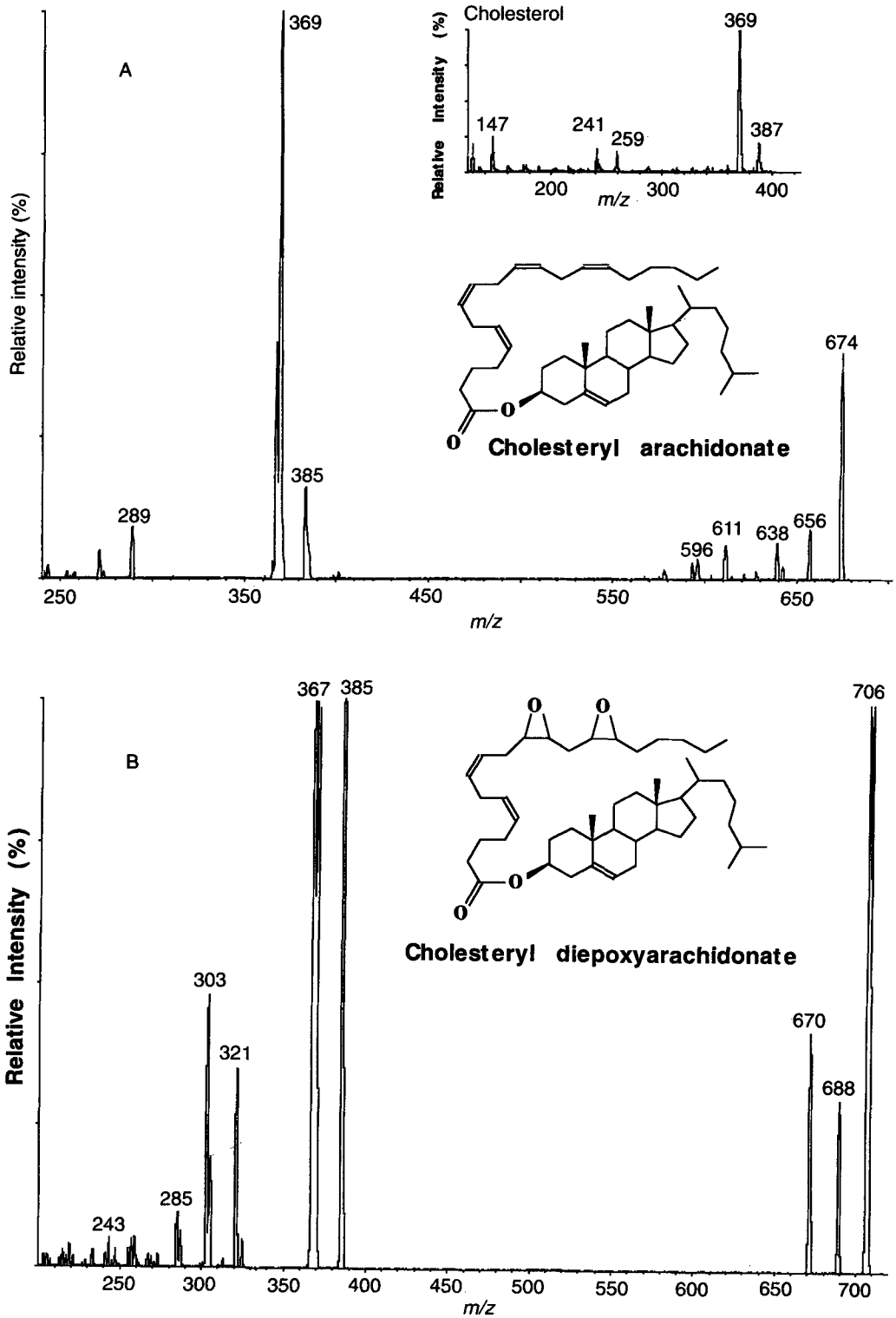


FIG. 3. A. Positive ion electrospray tandem mass spectrum of cholesterol (inset) and cholesteryl arachidonate. B. Positive ion electrospray tandem mass spectrum of diepoxy cholesteryl arachidonate. There are a number of positional and geometric isomers contributing to this spectrum, the structure of only one of which is depicted.

rivatives was most active against the human enzyme, with 500 nM inhibiting substrate hydrolysis by almost 80%.

Cholesterol, cholesteryl arachidonate, CAE, and CA polyols [CAE hydrolyzed by the method of Nourooz-Zadeh *et al.* (32)], were tested to determine their lowest inhibitory concentrations against *L. giganteum* PLA_2 and eluted from the DEAE-Sephacel column (100 mM NaCl/fraction 5 and 800 mM NaCl/fraction 2). The mixture of CAE was by far the most potent inhibitor, with >70% inhibition at levels as low as 6 nM. Inhibition comparable to that found using CAE was obtained using cholesterol, cholesteryl arachidonate, and the mixture of cholesteryl arachidonate diols (obtained by chemical hydrolysis of the CAE mixture) at concentrations of 600 μM, 70 μM, and 600 μM, respectively. The polyol mixture inhibited the activity of bovine pancreas PLA_2 by over 90% at a concentration of 3 μM.

DISCUSSION

We have demonstrated a correlation between ability of certain sterols to induce oosporogenesis and selectively inhibit *L. giganteum* PLA_2 enzymes. Lack of effect by sterols on some enzymes was expected since this parasite, which completely digests its mosquito larval hosts, must have a variety of lipolytic enzymes. Some of these would be constitutively expressed, nonspecific lipases, while others—those more likely to be under regulatory control—would be involved in specific developmental processes. Using mouse fibroblast cell membranes as substrates, it had previously been shown that sterol depletion enhanced metabolism of phosphatidylcholine (but not phosphatidylethanolamine) by PLA_2 present in β-bungarotoxin (36), but we found no effect of cholesterol on the activity of enzymes from bovine pancreas or snake venom.

This report is the first to show *in vitro* sterol regulation of a specific class of enzyme since sterol induction of reproduction by *Pythium and Phytophthora* was first described over three decades ago (2,3,37). Showing a physiological role for sterols and steryl derivatives in regulating PLA_2 and (a)sexual reproduction by this parasite would require a concerted research effort, that would include determination of: substrate specificity of acyltransferases, epoxidases (cytochromes P450), and epoxide hydrolases; developmental regulation of SE and PLA_2 synthesis; and subcellular colocalization of SE and specific PLA_2 that are subject to (epoxy) SE regulation.

The SE FAME composition of *L. giganteum* did not mimic either the whole cell or membrane composition. This preliminary observation suggests that this lipid class, generally considered to be metabolically inert (38,39), may have physiological functions in addition to storage and lipid transport.

Interfacial activation of PLA_2, and most other enzymes involved in lipid metabolism, are operationally defined as enzyme activity directed toward substrates at an organized interface (40). In addition to enzyme and substrate concentrations, kinetic analysis (and screening for inhibition) of these enzymes must also consider the organization of the lipid aggregate and related factors that affect enzyme binding to the interface (24,41,42). At least for pharmacologically and biochemically useful compounds, only active site-directed inhibitors are desirable. Compounds that act by restricting access of enzyme to substrate by binding to the vesicle surface are of little interest.

It is possible that the effects on *L. giganteum* PLA_2 of cholesterol and other sterols and steroids listed in Figure 1 were due to this type of nonspecific interference. This conclusion is based on the relatively high concentration of steroid needed to inhibit substrate hydrolysis. It is generally assumed that structural features of sterols necessary for them to interact with phospholipids include a planar ring sytem, a hydrophobic side-chain with or without a C-24 alkyl substituent, and a free 3β-OH group (43). Interpretation of our results is complicated by the fact that our assay used a DMSO-based vesicle system. It is likely that all of the steroids and sterols examined were solubilized by DMSO, although it could be argued that only those compounds meeting the structural criteria listed above associated with the GPL substrate per se. Inactive compounds such as cholestane and cholesteryl epoxide could preferentially partition into DMSO-rich areas of the vesicles, having minimal effect on enzyme access to substrate. This interpretation, however, does not explain why desmosterol, which also meets the criteria for interacting with GPL, was ineffective in inhibiting parasite PLA_2. Partitioning of the relatively hydrophilic ecdysteroids during the PLA_2 assays is difficult to predict, but these compounds were inactive both in the induction of oosporogenesis and in PLA_2 inhibition. In any case the high concentration of sterol needed for inhibition does not preclude a physiological role for cholesterol and related sterols in regulating this enzyme *in vivo*, since in some membranes cholesterol can make up over 50 mol% of total lipid.

The case for specific interaction with the enzyme becomes stronger as the results with the SE are considered. First, since all the compounds we examined had fatty acids in the C-3 position, the requirement for a free 3β-OH group is not met. As before, it is probable that DMSO-based vesicles will incorporate many classes of lipids, including SE, but preferential association of the inhibitors with the glycerolipid substrate is not likely. Second, we documented specific inhibition of enzymes from different biological sources by different steroids. If these compounds were acting primarily by binding to vesicles and thereby interfering with enzyme access to substrate, it would be expected that all PLA_2 would be similarly affected. It is difficult to explain why, for example, cholesteryl arachidonate but not cholesteryl linolenate inhibited *L. giganteum* PLA_2 if these compounds were only acting by nonspecific interference with enzyme binding to vesicles. Third, as we proceeded to evaluation of the steryl epoxy fatty acid derivatives, inhibition (or in some cases activation) of substrate hydrolysis occurred using nanomolar concentrations of these compounds. Since the substrate concentration is nearly 10 μM, it is not likely that nanomolar inhibitor concentrations would have the observed effects on enzyme activity. Kinetic analyses of PLA_2 are best performed using

assays in the scooting mode and monitored by the pH-stat method (44). Our radiometric assay is not amenable to this type of analysis, so we cannot support our conclusions with kinetic data.

Triterpenoids isolated by Jain *et al.* (24) from peppercorn berries that inhibit PLA_2 activity bear the closest structural resemblance of any described inhibitors to the steryl derivatives described here. The two inhibitory compounds that they describe, which have either a hydroxyl or a carbonyl group at C-3, are characterized by a terminal carboxyl group on the alkyl moiety attached to C-17, which appeared to be in contact with the catalytic site. Unlike those compounds, the SE evaluated in this investigation are not amphipathic. As concluded by Jain *et al.* (24), who used x-ray crystallographic data generated for native enzymes and those bound to various inhibitors (45–47), it is possible that the rigid tetracyclic nucleus of these lipids interacts with the hydrophobic region bordering the cavity that leads to the active site. In order to explain the specificity of inhibition demonstrated in this investigation, it must be assumed that the fatty acyl moiety interacts directly with the catalytic site. The size of the active site pocket in the different classes of PLA_2 has been invoked as the explanation for specificity or lack thereof for hydrolysis of certain fatty acids (48), and may explain preferential inhibition of enzymes from different organisms by different steryl derivatives. Activation of the human 85 kDa enzyme by CAE is complemented by preferential hydrolysis of arachidonic acid in GPL by this enzyme. Since none of the steryl epoxy fatty acids were completely purified, it is not possible to delineate the precise structural features required for inhibition.

Introduction of epoxide or hydroxyl groups into the esterified fatty acyl chain greatly enhanced their inhibitory activity. This is in apparent contrast to the increased activity of PLA_2 enzymes from several organisms documented when the phospholipid substrate included peroxidized lipids (49–52). The structure of the oxidized moieties was not examined in those studies, although it is probable that at least some hydroxyl and epoxide groups were produced by nonenzymatic dehydration of the hydroperoxides.

Lipoxygenase or related fatty acid oxygenase activity has been demonstrated to regulate sexual reproduction by *L. giganteum* (53) and related Oomycetes (54–56). We have documented synthesis of cholesteryl epoxy arachidonate by *L. giganteum*, and oxygenation of SE could be involved in regulating growth and reproduction by these organisms.

Several useful observations emerged from this investigation of sterol-mediated PLA_2 activity. SE may not be as metabolically inert as previously assumed, and the length of the fatty acyl moiety differentially regulates PLA_2 activity. Epoxidation of fatty acid SE can significantly alter biochemical activity of these compounds. The discovery of a new class of inhibitor for these enzymes, with selective activity against PLA_2 from different organisms, has possible implications for treatment of inflammatory diseases.

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Alk-1-enylacyl, Alkylacyl, and Diacyl Subclasses of Native Ethanolamine and Choline Glycerophospholipids Can Be Quantified Directly by Phosphorus-31 NMR in Solution[1]

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ABSTRACT: We show that phosphorus-31 nuclear magnetic resonance spectroscopy can be used to distinguish and to quantify the alk-1-enylacyl, alkylacyl, and diacyl glycerophosphoethanolamine (GPE) subclasses, and the respective glycerophosphocholine (GPC) subclasses, in their native form without prior degradation or derivatization, provided the phospholipids are observed in the nonaggregated state. Monomeric phospholipid distribution is ascertained by recording the spectra, after removal of metal ions, on $CDCl_3/CD_3OD/D_2O$ (50:50:15, by vol) solutions. The utility of this approach is exemplified for the ethanolamine glycerophospholipids (EPL) from bovine brain and the choline glycerophospholipids (CPL) from bovine heart. Sharp and well-resolved resonances are obtained for alkylacylGPE (+0.395 ppm; re 1% H_3PO_4), alkenylacylGPE (+0.353 ppm), and diacylGPE (+0.315 ppm), and for alkylacylGPC (–0.383 ppm), alkenylacylGPC (–0.436 ppm) and diacylGPC (–0.451 ppm). Integrated peak areas are shown to closely correlate with dose. Accurate quantitation of EPL and CPL subclasses at submicromolar levels can further be facilitated by use of synthetic dialkylGPE (+0.602 ppm) and dialkylGPC (–0.196 ppm) as internal standards. The method is simple, rapid, sensitive and reproducible, and permits the complete resolution and direct quantitation of all ethanolamine and choline glycerophospholipid subclasses quite independent of fatty chain length and degree of unsaturation.
Lipids 31, 1189–1195 (1996).

Phosphatidylethanolamines (PE) and phosphatidylcholines (PC) from natural sources often contain the respective alk-1-enylacyl and alkylacyl subclasses in addition to the diacyl species (for reviews, see Refs. 2–4). In fact, in some tissues, including brain (5–8), nerve (9) or heart (5–7, 10,11), the alk-1-enylacyl glycerophosphoethanolamine (GPE) or glycerophosphocholine (GPC) species can be more abundant than diacylGPE or diacylGPC. However, it has generally not been possible either to separate the native alk-1-enylacyl, alkylacyl and diacyl ethanolamine glycerophospholipid (EPL) or choline glycerophospholipid (CPL) subclasses from each other, or to quantitate the intact subclasses in the presence of each other.

Current procedures for the analysis of the alkenylacyl, alkylacyl, and diacyl GPE and GPC subclasses in biological samples typically involve acidic, alkaline or phospholipase-catalyzed hydrolysis (5,10,12–15), acid-catalyzed transmethylation (16), or $LiAlH_4$ reduction (17,18) followed by characterization of the long-chain moieties by gas chromatographic techniques (for a review, see Ref. 19). Alternatively, the EPL and CPL subclasses can be resolved as the respective diradylglycerol derivatives which are obtained by phospholipase C catalyzed removal of the polar phosphobase headgroup (14,20,21) and by replacing it with a less polar acetyl (8,14,20,22), benzoyl (23,24), or 3,5-dinitrobenzoyl (25–28) function. The nonpolar diradylglycerol derivatives can then be resolved by thin-layer chromatography (TLC; Refs. 14,20,23), gas–liquid chromatography (22), or high-performance liquid chromatography (8,23–28), and further assayed or characterized by spectroscopic techniques (15,23,29).

We present here an alternative experimental approach by which the native alk-1-enylacyl, alkylacyl and diacyl GPE and GPC subclasses can be distinguished from each other and can be quantified directly on natural EPL and CPL fractions without prior degradation or derivatization. The method is based on the observation that phosphorus-31 nuclear magnetic resonance (NMR) gives distinct and well-resolved signals for each of the EPL and CPL subclasses, provided that intermolecular interactions are minimized through appropriate choice of the solvent medium.

MATERIALS AND METHODS

Materials. Solvents used were freshly distilled from a glass apparatus. Deuterated solvents were purchased from Cam-

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Abbreviations: CPL, choline glycerophospholipids; EPL, ethanolamine glycerophospholipids; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TLC, thin-layer chromatography.

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bridge Isotope Laboratories (Andover, MA). EPL from bovine brain (brain PE fraction) and CPL from bovine heart (heart PC fraction) were obtained from Avanti Polar Lipids (Alabaster, AL). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine used as standards were also from Avanti. Ether lipid standards were synthesized in our laboratory following established procedures (30,31). *rac*-1,2-Di-*O*-octadecylglycero-3-phosphoethanolamine used as dialkylGPE standard was synthesized from dioctadecyl glycerol ether (32) using the Hirt and Berchtold (33) phosphorylation procedure; *rac*-1,2-di-*O*-hexadecylglycero-3-phosphocholine used as dialkylGPC standard was prepared from dihexadecyl glycerol ether (32) according to Hansen *et al.* (34). All standards used were pure as judged by TLC (see below), and their structures were verified by ^{13}C NMR on $CDCl_3/CD_3OD/D_2O$ (50:50:15, by vol) solutions (35) and comparison with published data (34,36,37).

Chromatography and sample preparation . Phospholipids were resolved by TLC on 0.5-mm layers of Silica Gel H (Merck, Darmstadt, Germany) using $CHCl_3/CH_3OH/H_2O$ (65:35:8, by vol) as developing solvent. TLC plates were predeveloped in methanol, dried, and activated at 120°C for 15 min prior to applying the lipid samples. For analytical purposes, fractions were made visible by spraying with concentrated sulfuric acid/CH_3OH (1:1, vol/vol) followed by charring at 190°C. In preparative work, lipid fractions were localized by gently blowing iodine vapors (by means of a Pasteur pipette containing a few iodine crystals) over the lanes of suitable standards that were typically run along both edges of the plate. Residual iodine was removed under a stream of nitrogen, and lipid bands were immediately scraped off the plate. Phospholipids were eluted with $CHCl_3/CH_3OH/H_2O$ (50:40:10, by vol), and the adsorbent was separated by filtration through a 0.45-μm Acrodisc (13CR; Gelman Sciences, Inc., Ann Arbor, MI) and washed two to three times with fresh solvent. To remove metal ions (38) from the combined eluates, the solvent ratio was adjusted to arrive at a Folch mixture (39) in which $CHCl_3$ and CH_3OH were present at a 2:1 (vol/vol) ratio with the water phase (20% of $CHCl_3/CH_3OH$ volume) consisting of 10 mM EDTA in 2 mM Tris buffer (pH 8), and the mixture was vortexed for 30 s. Upon separation of the layers, the upper phase was reextracted with the appropriate amount of fresh Folch lower phase, and the combined lower phase lipid extracts were evaporated under a stream of nitrogen prior to NMR analysis. Total phospholipid contents were determined by inorganic phosphorus assay (40).

Phosphorus-31 NMR measurements. ^{31}P NMR spectra were recorded at 121.42 MHz on a Varian Unity 300 instrument (Varian Associates, Palo Alto, CA) using a 5-mm variable temperature probe. Standard single-pulse experiments entailed a 90° pulse of 10 μs, an acquisition time of 3.2 s, and a pulse delay of 16 s (T_1 ~3 s), with the decoupler gated *on* during acquisition only. The spectral width was 430 Hz, and 2752 datapoints were collected. The number of transients ranged from 16 to 3200, depending on the phospholipid concentration. Data were zero-filled and Fourier-transformed without applying exponential linebroadening which permitted a spectral resolution of 0.1 Hz. Spectra were referenced relative to 1% H_3PO_4 which was sealed into a 1-mm i.d. glass capillary that was concentrically placed in the NMR sample tube to serve as external standard. The 1% H_3PO_4 standard (0.00 ppm) was chosen in lieu of the more common 85% H_3PO_4 reference (–0.48 ppm) to keep the signal intensity of the standard commensurate with the phospholipid signal intensities observed on the small analytical samples used. Spectra were recorded at 25 ± 0.1°C on freshly prepared samples dissolved in $CDCl_3/CD_3OD/D_2O$ (50:50:15, by vol), using $CDCl_3$, CD_3OD, and D_2O that had been purged of dissolved oxygen by a stream of nitrogen to minimize linebroadening. Peak assignments were verified based on alkenylacyl/alkylacyl conversion by hydrogenation and on acid-catalyzed hydrolysis of the alkenylacyl species, and were aided by the use of synthetic alkylacyl and diacyl phospholipid standards. Peak areas were measured by digital integration.

RESULTS

Figure 1 shows the high-resolution phosphorus-31 NMR spectra (121.42 MHz) which we obtained on a PE fraction from bovine brain (Fig. 1, left panel) and on a PC fraction from bovine heart (Fig. 1, right panel). Spectra were recorded after isolating the ethanolamine and the choline glycerophospholipid fractions by TLC, removing metal ions by EDTA treatment, and upon dispersing the samples in deuterated chloroform/methanol/water (50:50:15, by vol). Sharp and well-resolved resonances are seen for alkylacylGPE (+0.395 ppm), alkenylacylGPE (+0.353 ppm), and diacylGPE (+0.315 ppm), with all the signals located downfield from 1% H_3PO_4, and for alkylacylGPC (–0.383 ppm), alkenylacylGPC (–0.436 ppm), and diacylGPC (–0.451 ppm), with all the signals located upfield from 1% H_3PO_4.

Figure 1 demonstrates that useful spectra can be obtained within a reasonable time (64 transients; ~20 min) on lipid samples of moderate size (10 μmol; ~7 mg). The spectra could easily be improved by increasing sample size or total acquisition time. Although the signal-to-noise ratios of these spectra were not optimized, the resonances are actually quite sharp with apparent linewidths at halfheight ($\nu_{1/2}$) of about 1.7 to 1.8 Hz for the EPL signals, and about 0.5 to 0.6 Hz for the CPL signals. The somewhat greater apparent linewidths at halfheight of the EPL signals can be attributed to minute chemical shift differences between respective diradylGPE species bearing saturated and unsaturated aliphatic chains. Such "finesplitting" is not indicated for the CPL signals (Malewicz, B., and Baumann, W.J., unpublished data).

Because the chemical shift differences between the alkylacyl, alkenylacyl, and diacyl GPE signals are quite small (i.e., 0.038-0.042 ppm; ~5 Hz), and are even smaller between alkenylacylGPC and diacylGPC (0.015 ppm; ~1.8 Hz), it is crucial that phosphorus linewidth is minimized and signal

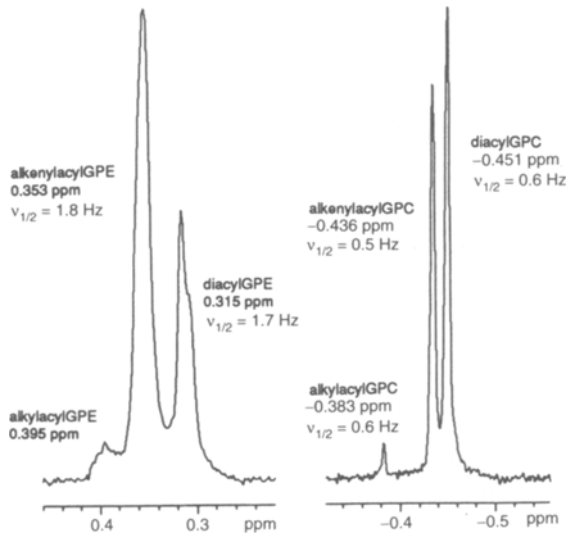


FIG. 1. ^{31}P nuclear magnetic resonance spectra (121.42 MHz) of the ethanolamine glycerophospholipids (EPL) from bovine brain (left panel) and the choline glycerophospholipids (CPL) from bovine heart (right panel). The EPL and CPL fractions were isolated by silicic acid thin-layer chromatography, using $CHCl_3/CH_3OH/H_2O$ (65:35:8, by vol) as developing solvent, and eluted from the adsorbent with $CHCl_3/CH_3OH/H_2O$ (50:40:10, by vol). Metal ions were removed by EDTA treatment, and spectra were recorded on samples (10 μmol each, 64 transients) dissolved in $CDCl_3/CD_3OD/D_2O$ (50:50:15, by vol). Chemical shifts are given in ppm relative to 1% H_3PO_4 (0.0 ppm) which served as external standard. Well-resolved narrow signals ($\nu_{1/2}$ 0.5–1.8 Hz) are observed for each of the alkylacyl, alkenylacyl and diacyl EPL and CPL subclasses.

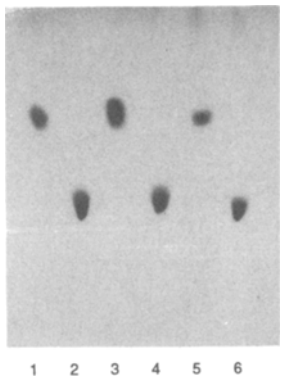


FIG. 2. Thin-layer chromatogram illustrating the relative migration rates of various ethanolamine and choline glycerophospholipids on Silica Gel H using $CHCl_3/CH_3OH/H_2O$ (65:35:8, by vol) as developing solvent. The fractions are designated as: **1**, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; **2**, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; **3**, ethanolamine glycerophospholipid fraction from bovine brain; **4**, choline glycerophospholipid fraction from bovine heart; **5**, *rac*-1,2-di-*O*-octadecylglycero-3-phosphoethanolamine; and **6**, *rac*-1,2-di-*O*-hexadecylglycero-3-phosphocholine. Phospholipid fractions were made visible by charring after spraying with sulfuric acid/CH_3OH (1:1, vol/vol).

resolution optimized by observing the phospholipid molecules in the nonaggregated state.

For practical purposes, phospholipid subclass analyses by ^{31}P NMR are best done on isolated ethanolamine and choline glycerophospholipid fractions as they are readily obtained, for example, by silicic acid chromatography on a TLC plate. Figure 2 illustrates the migration rates in TLC of the brain EPL fraction (**3**) and the heart CPL fraction (**4**) relative to 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (**1**) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (**2**) with chloroform/methanol/water (65:35:8, by vol) as the developing solvent. Recording the spectra on isolated ethanolamine and choline glycerophospholipid fractions ensures that the respective EPL and CPL subclass resonances are observed without possible interference from other phospholipid signals. This is particularly important for the analysis of the EPL subclasses because the phosphatidylserine signal, for example, can overlap with the diacylGPE resonance (data not shown). However, if so desired, the EPL and CPL subclasses can reliably be assayed in the presence of each other.

Quantitative information can be obtained from the spectra by simple integration of peak areas. Because the phospholipids under consideration here each contain only a single phosphate group, the ratios of integrated peak areas do directly translate into mole percentages. No corrections need to be made for variations in chain length or degree of unsaturation.

Thus, digital integration of the EPL subclass resonances (Fig. 1, left panel; $n = 3$) showed that the ethanolamine glycerophospholipid fraction from bovine brain consisted of 64.0 ± 1.2 mol% alkenylacylGPE, 31.8 ± 0.5 mol% diacylGPE, and 4.2 ± 1.3 mol% alkylacylGPE, which is in good agreement with the data obtained by Blank *et al.* (63.5 ± 1.2, 30.6 ± 1.3, and 5.9 ± 1.2 mol%, respectively; see Ref. 23) who analyzed the EPL subclasses as diradylglycerol benzoates upon phospholipase C hydrolysis of bovine brain EPL. Similarly, integration of the CPL subclass resonances (Fig. 1, right panel; $n = 3$) showed that the choline glycerophospholipid fraction from bovine heart consisted of 55.5 ± 1.3 mol% diacylGPC, 40.9 ± 1.0 mol% alkenylacylGPC, and 3.6 ± 0.4 mol% alkylacylGPC, which compares well with the data obtained by Pugh *et al.* (57, 39, and 3.6 mol%, respectively; see Ref. 10) upon acid-catalyzed and alkaline hydrolyses of bovine heart CPL. If, in addition, total phospholipid phosphorus (40) is assayed, the relative mole percentages obtained by digital integration of peak areas can then, of course, also be converted into absolute concentrations.

Absolute subclass quantitation can directly, and more conveniently, be achieved, however, by phosphorus NMR by use of quantitative internal standards. The synthetic dialkyl glycerol ether analogs of PE and PC, i.e., dialkylGPE and dialkylGPC, lend themselves particularly well for this purpose. Both, dialkylGPE and dialkylGPC give rise to distinct signals in phosphorus NMR, with the dialkylGPE resonance appearing at 0.602 ppm (see Fig. 3, insert, **St**), well separated from alkylacylGPE (**1**), alkenylacylGPE (**2**), and diacylGPE (**3**), and the dialkylGPC resonance appearing at −0.196 ppm (see Fig. 4, insert, **St**), well separated from alkylacylGPC (**1**), alkenylacylGPC (**2**), and diacylGPC (**3**). This makes it possi-

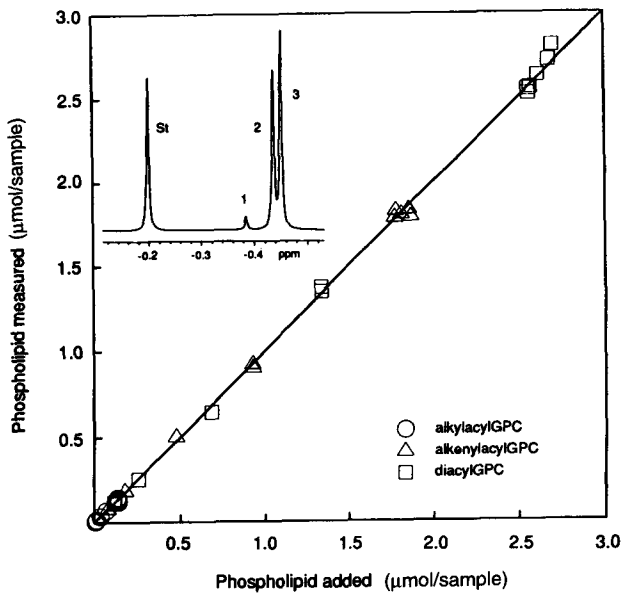


FIG. 3. Quantitation by ^{31}P nuclear magnetic resonance spectroscopy of the alkylacyl, alkenylacyl, and diacyl ethanolamine glycerophospholipid subclasses illustrated for bovine brain EPL. The spectral insert shows the resolved resonances of alkylacylGPE (**1**), alkenylacylGPE (**2**), and diacylGPE (**3**) relative to *rac*-1,2-di-*O*-octadecylglycero-3-phosphoethanolamine which was added as quantitative internal standard (**St**) prior to thin-layer chromatographic isolation of the EPL fraction (see the Materials and Methods section). The plot illustrates the excellent correlation that exists between EPL dose, as measured by inorganic phosphorus assay, and the respective EPL subclass levels determined by phosphorus NMR based on integrated peak areas relative to the dialkylGPE standard (500 nmol).

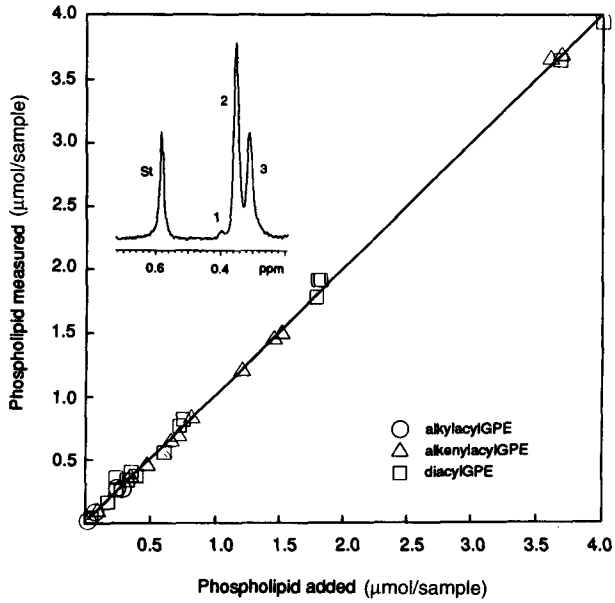


FIG. 4. Quantitation by ^{31}P nuclear magnetic resonance spectroscopy of the alkylacyl, alkenylacyl, and diacyl choline glycophospholipid subclasses illustrated for bovine heart CPL. The spectral insert shows the resolved resonances of alkylacylGPC (**1**), alkenylacylGPC (**2**), and diacylGPC (**3**) relative to *rac*-1,2-di-*O*-hexadecylglycero-3-phosphocholine which was added as quantitative internal standard (**St**) prior to thin-layer chromatographic isolation of the CPL fraction (see the Materials and Methods section). The plot illustrates the excellent correlation that exists between CPL dose, as measured by inorganic phosphorus assay, and the respective CPL subclass levels determined by phosphorus NMR based on integrated peak areas relative to the dialkylGPC standard (500 nmol).

ble to quantitate each EPL and CPL subclass by direct comparison of its integrated peak area with that of the respective internal diether phospholipid standard.

The accuracy and sensitivity of the assay are illustrated for alkylacylGPE (**1**), alkenylacylGPE (**2**), and diacylGPE (**3**) relative to dialkylGPE (**St**) in Figure 3, and for alkylacylGPC (**1**), alkenylacylGPC (**2**), and diacylGPC (**3**) relative to dialkylGPC (**St**) in Figure 4. The plots show the excellent correlation that exists over a wide range of concentrations between phospholipid dose, as measured by inorganic phosphorus assay, and the respective phospholipid subclass levels as determined by phosphorus-31 NMR based on integrated peak areas relative to those of dialkylGPE and dialkylGPC (500 nmol) used as quantitative internal standards. Low micromolar quantities of phospholipid subclass can be assayed rapidly (64–640 transients) with an error of less than ±5%. Given sufficient acquisition time (3200 transients), even samples as small as 10 nmol of CPL subclass or 50 nmol of EPL subclass can be quantitated with an error of less than ±10%.

Use of the diether glycerophospholipids as internal standards has the additional advantage that dialkylGPE (**5**) and dialkylGPC (**6**) migrate in silicic acid chromatography together with the respective alkenylacyl, alkylacyl, and diacyl ethanolamine (**3**) and choline (**4**) glycerophospholipids (see Fig. 2). Hence, when added to a lipid extract, the diether analogs are isolated together with the respective natural EPL and CPL fractions and thus can also account for any losses that occur in the course of phospholipid isolation (see Figs. 3 and 4).

DISCUSSION

Observance of isotropic phospholipid phosphorus resonances that can be interpreted in quantitative terms requires that line-broadening is minimized by measuring the spectra on phospholipids in the nonaggregated state. Yet, most phospholipids because of their amphiphilic nature resist true solubilization in polar or in nonpolar solvents where they form molecular aggregates, such as liposomes or reverse micelles. We have previously shown that nonaggregation of phospholipids in a given medium is indicated in carbon-13 NMR by the observance of maximal two-bond and three-bond carbon–phosphorus couplings (J_{CP}; Ref. 36), and in the case of choline phospholipids by the appearance of well-defined ^{13}C-^{14}N couplings (J_{CN}) and by the magnitude of the ^{14}N quadrupolar relaxations (^{14}N T_1; Ref. 35). Based on these criteria, chloroform/methanol/water (50:50:15, by vol) has evolved as the medium of choice in which a wide range of natural phospholipids can be observed in the nonaggregated state (34–37). We

have utilized this observation in the present study to resolve and to analyze by phosphorus NMR all alkenylacyl, alkylacyl, and diacyl subclasses of ethanolamine glycerophospholipids from bovine brain and choline glycerophospholipids from bovine heart.

Two different approaches have previously been used to characterize phospholipid dispersions by phosphorus NMR. By the first approach, phospholipids are dispersed as mixed micelles with bile acids or other detergents (41,42). Although good resolution between phospholipid class and species resonances can be achieved with defined compounds on model systems (41–43), the detergent mixed micelle approach has not been proven practical for the actual analysis of more complex natural phospholipid mixtures (41,44,45). The resolution of alkylacyl, alkenylacyl, and diacyl GPC or GPE subclass resonances has not been accomplished using the detergent micelle technique (44). By the second approach, phospholipids are dispersed in a two-phase, Folch-like solvent mixture (39) supplemented with chelating agent and monovalent ions (e.g., chloroform/methanol/0.2 M aqueous cesium or potassium EDTA, 10:4:1, by vol), and the spectra are recorded on the organic lower phase in the presence of the aqueous upper phase (46). However, in the two-phase system, signal separations and chemical shifts can vary substantially with the make-up of the lower phase, dependent on lipid source and lipid concentration, which makes peak assignments tenuous (47–49). Particular confusion exists in the literature in regard to the resolution and assignment of alkylacyl, alkenylacyl, and diacyl GPC and GPE resonances in spectra obtained on complex phospholipid mixtures. Although it was first suggested that the alkenylacylGPC and diacylGPC resonances can be resolved by the two-phase approach (46), others have later shown that the initial claim was based on the mistaken identification of alkylacylGPC as alkenylacylGPC (49,50). Yet, misassignment of alkylacylGPC as alkenylacylGPC is still prevalent in the literature (e.g., 51–53), and alkenylacylGPC and diacylGPC have still not been resolved. Moreover, in the two-phase system the alkenylacylGPE and diacylGPE resonances do frequently overlap with each other and with other phospholipid signals (49), and the alkylacylGPE resonance has not been identified (e.g., 49,54,55).

The present study has shown that phosphorus NMR can resolve all native alkenylacyl, alkylacyl and diacyl glycerophosphoethanolamine and glycerophosphocholine subclasses if monomeric phospholipid distribution is ensured by use of a single-phase chloroform/methanol/water (50:50:15, by vol) system as the solvent medium. Characteristic and narrow phosphorus resonances are thus obtained ($\nu_{1/2}$ 0.5–1.8 Hz) for each of the ethanolamine and choline glycerophospholipid subclasses irrespective of lipid source and lipid concentration, and essentially independent of fatty chain length and degree of unsaturation. Simple integration of peak areas gives relative molar subclass concentrations. Absolute concentrations can directly be measured by use of the respective diether phospholipids, dialkylGPE and dialkylGPC, as quantitative internal standards. The method is simple, rapid, sensitive, and reproducible and permits the complete resolution and direct quantitation of all ethanolamine and choline glycerophospholipid subclasses without prior degradation or derivatization.

Phospholipid subclass quantitation by NMR has, of course, the further advantage that the lipid samples are conserved and thus can still subsequently be used for molecular species and fatty chain analyses, if so desired.

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Effect of Sample Storage on Quantitation of Lipoprotein(a) by an Enzyme-Linked Immunosorbent Assay

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ABSTRACT: This study evaluated the effect of storage on the quantitation of lipoprotein (Lp)(a) in 25 serum samples. Aliquots of serum were stored for up to three years at either −20°C or −70°C and Lp(a) subsequently analyzed using an enzyme-linked immunosorbent assay kit. Concentrations of Lp(a) declined during storage, and the temperatures employed elicited significantly different ($P < 0.05$) values within 12 mon which further diverged during three years of storage. Compared to baseline values, significant decreases ($P < 0.05$) in Lp(a) levels were evident after six months of storage at −20°C with apparent losses (geometric mean) reaching 36.9% (95% confidence interval: 30.9%, 42.9%) after three years. Similarly, significantly lower ($P < 0.05$) Lp(a) values were recorded after six months of storage at −70°C and at three years the decrease (geometric mean) was 19.1% (95% confidence interval: 14.3%, 24.0%). The losses, after three years, in terms of the arithmetic mean were 53.5 and 26.2% at −20 and −70°C, respectively. Phenotype analysis suggested that large isoforms are more susceptible to degradation than smaller moieties. This may be related to the observation that apparent losses are reduced in samples containing over 8 mg/dL Lp(a). Nevertheless, Lp(a) levels in stored samples retained a strong correlation with the baseline values. These results must be considered specific for the storage conditions and analytical procedures employed.
Lipids 31, 1197–1203 (1996).

Lipoprotein (Lp)(a) is a predominantly cholesterol-rich lipoprotein similar in structure to low density lipoprotein but possessing an apolipoprotein (apo)(a) moiety linked to $apoB_{100}$ *via* a disulfide bridge (1,2). A triglyceride-rich Lp(a) particle containing apoE also occurs but represents only 10 to 20% of total plasma Lp(a) (3,4).

Apo(a) exists in over 30 isoforms which range in molecular weight from 200 to 838 kDa (5,6). Each isoform contains one kringle 5 moiety, so named because of its 85% homology with the corresponding domain in plasminogen. The isoforms differ in their number of kringle 4s moieties. The kringle 4s can be further classified into ten distinct forms (types 1 to 10). One copy each of type 1 and of types 3 to 10 occurs in all isoforms but variable numbers of the type 2 moiety accounts for the heterogeneity of apo(a) (5,7,8). The large number of isoforms complicates development of a standardized assay procedure for Lp(a) and hinders evaluation of its physiological role (9).

Serum Lp(a) concentrations range from less than 1 mg/dL to over 100 mg/dL. These quantities are highly skewed toward high values in Caucasians and Asians but less so in Blacks who tend to have higher levels of Lp(a) (10–12). An inverse relationship exists between apo(a) isoform size and Lp(a) concentration (13,14).

Study of Lp(a) is prompted by reports suggesting an association between Lp(a) levels and vascular disease independent of other lipoproteins and risk factors (15–22). The action of Lp(a) is suggested to involve both atherogenic and thrombotic mechanisms (23,24). Nevertheless, several studies question the association between Lp(a) concentrations and vascular disease (25–28). Various suggestions have been offered to account for these dissenting observations, including sample storage, group size, population differences, allele distribution, and duration of follow-up.

In this study, the effect of sample storage on Lp(a) measurements was evaluated. Samples were stored for up to three years, at either −20 or −70°C, prior to analysis using an ELISA. These results are needed for critical evaluation of long-term prospective and retrospective data.

EXPERIMENTAL PROCEDURES

Subjects. The study group consisted of 25 consecutive healthy employees (13 males, 12 females) of the University of Pittsburgh ranging in age from 25 to 43 yr.

Laboratory. Subjects were instructed to fast after 9:00 pm on the night before blood collection, which occurred between 8:30 and 11:00 a.m. Blood samples were collected into 15 mL plain red-top vacutainers (Becton Dickinson, Rutherford, NJ) and held at ambient temperature for 45 min. Serum was sepa-

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Abbreviations: Apo(a), apolipoprotein(a); Lp(a), lipoprotein(a); RID, radial immunodifusion.

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rated by centrifugation at 1500 × *g* for 15 min at 4°C. The serum was then divided into 14 aliquots of 0.5 mL and placed into 2-mL polypropylene screw-top 0-ring tubes (Sarstedt, Newton, NC). Seven 0.5 mL aliquots from each subject were stored at −20 and −70°C. No additives were added to the aliquots prior to storage, and no freezer breakdowns occurred during this study.

Lp(a) was measured using a commercially available ELISA kit (Strategic Diagnostics, Newark, DE; previously Terumo) that reports Lp(a) particle as mg/dL. In brief, serum (10 μL) with 2 mL of diluent containing protein stabilizers and thimerosal were incubated (60 min at room temperature) and rotated (120 rpm) in a covered (Laboratory Wrap; Fisher, Pittsburgh, PA) multicell plate containing monoclonal anti-apo(a) antibody bound to the well surfaces. This antibody reacts with an apo(a) Kringle 4-like epitope in all 33 isoforms tested (29). Unbound antigen was removed by washing with a buffered solution containing thimerosal and detergent. There were four washes in total, and the plate was rotated 180° after the second aspiration. Subsequently, a second incubation with a polyclonal anti-apo(a) antibody conjugated with horseradish peroxidase was undertaken (20 min at room temperature) with rotation (120 rpm) and covering (Laboratory Wrap). The plate was then washed, as above, and color development started by adding a fresh (<20 min) solution (100 μL) of hydrogen peroxide and 0-phenylenediamine. The reaction 10 min at room temperature with rotation at 120 rpm was stopped by adding 2 N sulfuric acid (50 μL) and the absorbance at 490 nm read within 15 min. Standards (0 to 80 mg/dL), pre-diluted controls, a laboratory pooled control, and duplicate samples were run with each assay. No edge effects were observed. The coefficient of variation between runs was 8%. The assay exhibits no cross-reactivity with plasminogen (100 mg/dL), low density lipoprotein (LDL) (200 mg/dL), high density lipoprotein (HDL) (400 mg/dL), or very low density lipoprotein (VLDL) (170 mg/dL). The analyses over the three-year period required the use of kits with different lot numbers (see legends to Tables 1 and 2). This was considered an unavoidable complication due to the lability of the kits. The standards, human plasma in a buffered solution with protein stabilizers and thimerosal, are reported to be stable during storage at 4°C for 20 mon. Strategic Diagnostics tests each lot number with nine serum and three plasma (EDTA) samples ranging in Lp(a) concentration from one to sixty mg/dL. For the lot number to be released for use, all values obtained must fall within two standard deviations of the accumulative mean. This procedure has been ongoing since 1989 and entails storage of the test samples at −80°C. The Strategic Diagnostics standard contains two isoforms, S_1 and S_3, that give bands of equal intensity on immunoblots (29). This nomenclature is based on that of Utermann *et al.* (13) and the S_1 and S_3 isoforms are assigned molecular masses of 520 kDa and 640 kDa, respectively. Additional documentation of the response of these two isoforms to the antibody is unavailable. Standardization of the assay is further complicated by the occurrence of over 30 isoforms (5,6), and Taddei-Peters *et al.* (30) report that this assay underestimates the concentration of the smaller isoforms. These limitations, however, do not affect the comparison of Lp(a) concentrations in the same groups of samples stored for different periods of time and at different temperatures, although it complicates attempts to interpret the influence of isoform size. An ELISA involving a monoclonal anti-apo(a) capture antibody and a polyclonal anti-apo lipoprotein β detection antibody has been reported to be insensitive to variation in apo(a) isoform size when reporting the data as moles/L (31).

Phenotype analysis of the apo(a) isoforms was done using SDS–agarose gel electrophoresis as described previously (32).

Statistical analysis. All statistical tests and calculations of rates of degradation were based upon the natural logarithm of the observed data. The percentage decline on the geometric scale refers to the difference between the mean of the logs at each time point and the mean of the log at baseline. This was done to correct for the highly skewed nature of the data. Statistical significance of observed temperature effects was assessed by running paired *t*-tests at each time point. Overall time effects and rates of degradation between time points were calculated by utilizing the Analysis of Variance and Covariance module of the BMDP Statistical Software package (BMDP Statistical Software, Inc., Los Angeles, CA). The difference in means of the transformed data and their 95% confidence intervals were calculated with a Bonferroni adjustment factor for multiple comparisons. It should be noted that this is a very conservative method which produces very wide confidence limits when calculation of degradation rates is performed. Confidence limits for the percentage decrease in the arithmetic means are not reported as the assumption of normally distributed data at each time point is clearly violated. Pearson correlation coefficients were calculated for the 1-, 2-, and 3-year data as related to the fresh samples.

RESULTS

Analysis of the 25 fresh samples indicated they had a mean Lp(a) concentration of 20.0 ± 21.6 (SD) mg/dL and a median of 9.6 mg/dL. The range was from 1.0 to 73.7 mg/dL, and twelve of the 25 subjects had plasma Lp(a) levels of over 10 mg/dL. Analysis of stored samples indicated that values declined during storage at both −20 and −70°C, and the decrease was significantly greater at the former temperature ($P < 0.05$) after one, two, and three years (Table 1). The differences between the −20 and −70°C results illustrate the effect of temperature as magnified by time and the distribution of the percentage decreases after three years (Fig. 1) emphasizes the greater losses observed at −20°C. Inspection of mean Lp(a) levels at each time point revealed an increase at three months. Although an apparent rise in Lp(a) concentration is possible, we believe that this elevation is due to analytical variation (see below). All results were evaluated with and without the three-month results, but for the ANOVA data reported in this paper the three month values were excluded. At both temper-

TABLE 1
Percentage change from Baseline Concentration After Storage at Either –20 or –70°C

	Temperature[a]			
	–20°C		–70°C	
	Geometric mean (CI)	Arthmetic mean	Geometric mean(CI)	Arithmetic mean
Storage time[b]				
One week	–1.7 (+4.3, –7.7)	–3.2	–0.8(+4.1,–5.6)	+0.1
Four weeks	–3.0(+2.9,–9.0)	–7.3	–2.4(+2.4,–7.2)	+2.6
Three months	+8.6[d]	+18.7	+1.8[d]	+9.0
Six months	–6.5(–0.5,–12.4)[c]	–9.2	–7.6(–2.8,–12.5)[c]	–7.4
One year	–12.1(–6.2,–18.1)[c]	–16.8	–5.1(–0.2,–9.9)[c]	–5.7
Two years	–22.7(–16.7,–28.7)[c]	–30.3	–8.1(–3.2,–12.9)[c]	–17.1
Three years	–36.9(–30.9,–42.9)[c]	–53.5	–19.1(–14.3,–24.0)[c]	–26.2

[a]The effect of temperature was significant for the one-, two-, and three-year results.
[b]Assay kit lot numbers used at each time point and number of months between analysis date and expiration date of kit given in parentheses: baseline (10 mon), one (10 mon), and four weeks (9 mon), NK2681; three months (5 mon), NM1081; six months (11 mon), PC2681; one year (9 mon; 7 mon), PG1681 (–20°C), PK2181 (–70°C); two years (15 mon), QK0181 and three years (1 mon), 104242.
[c]Significantly different from baseline values at $P < 0.05$.
[d]Confidence intervals not computed for three months time point (see text).

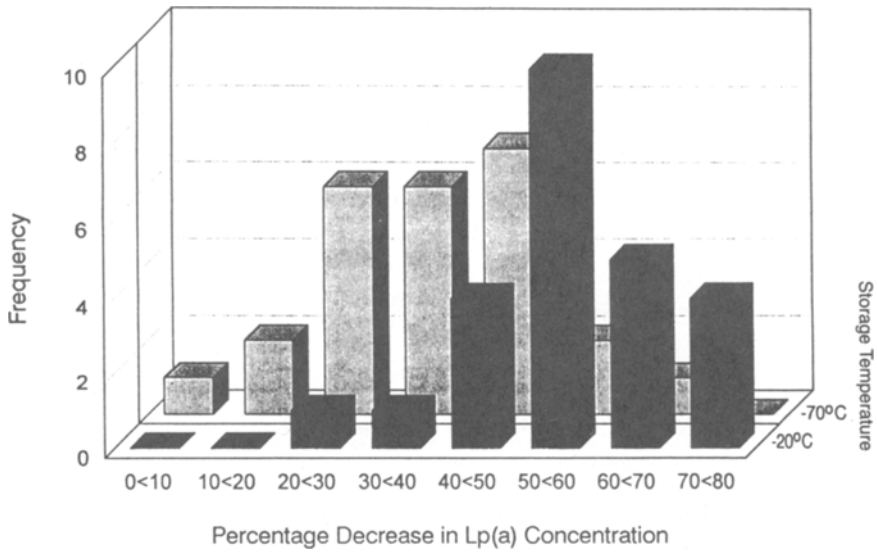


FIG. 1. Distribution of percentage decreases in lipoprotein (Lp)(a) concentration after storage for three years at either –20 or –70°C.

atures the first significant decrease from the baseline values was observed at the six-month time point. After three years the percentage decrease in the arithmetic mean was 53.5 and 26.2% for the –20 and –70°C samples, respectively. As noted in the methodology, confidence limits for the percentage decrease in the arithmetic means are not reported. At three years the percentage decrease in the geometric mean was 36.9% (95% CI: 30.9%, 42.9%) at –20°C and 19.1% (95% CI: 14.3%, 24.0%) at –70°C. Although absolute values decreased during storage, the values for stored samples retained a strong correlation with the concentrations of the baseline samples: r = 0.970 and 0.987 for the three years, –20 and –70°C, samples respectively (Fig. 2).

Although the 25 consecutive samples analyzed demonstrate the skewed distribution typical of general populations, the presence of specimens with low Lp(a) values complicates interpretation of the data. The precision of the analysis becomes a major consideration at low concentrations. Consequently the statistical analyses were repeated for the 15 samples with Lp(a) levels exceeding 8 mg/dL. The results (Table 2) indicate that the percentage decline in Lp(a) concentrations is less than that for the total sample. The decreases are not significant until one year and two years at –20 and –70°C, respectively. The effect of temperature is significant at all time points, except at six months.

DISCUSSION

Stability of the analyte is always a concern during studies involving analysis of stored samples. Loss of integrity may lead to spurious conclusions whereas stability promotes long-range prospective and retrospective studies. The literature involving Lp(a) stor-

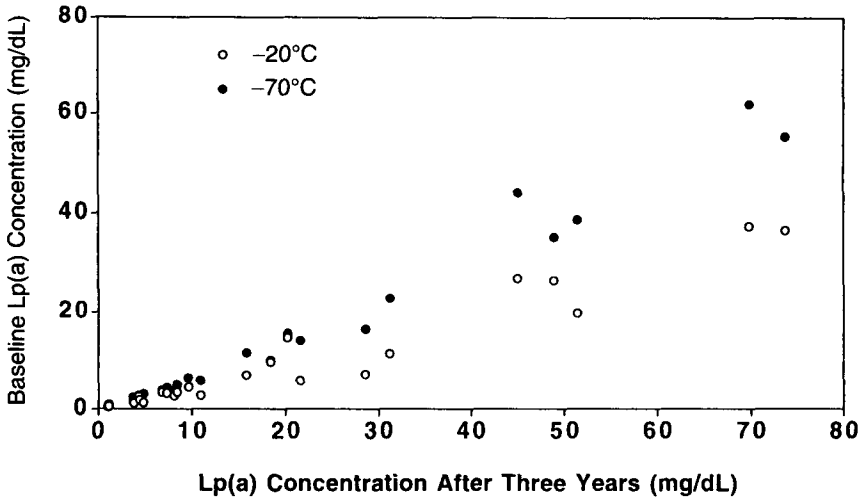


FIG. 2. Correlation of Lp(a) concentrations at baseline with values obtained after storage for three years at either –20°C ($r = 0.970$) or –70°C ($r = 0.987$). See Figure 1 for abbreviation.

TABLE 2
Percentage Change from Baseline Concentration in Samples with over 8 mg/dl of Lp(a) at Baseline After Storage at Either –20 or –70°C

	Temperature[a]			
	–20°C		–70°C	
	Geometric mean (CI)	Arithmetic mean	Geometric mean(CI)	Arithmetic mean
Storage time[b]				
One week	–1.5 (+4.0, –7.1)	–3.5	–0.2(+3.6,–4.0)	+0.2
Four weeks	–2.6(+3.0,–8.2)	–7.5	+0.2(+4.0,–3.6)	+3.7
Three months	+5.2[d]	+17.5	+2.4[d]	+9.4
Six months	–2.6(+3.0,–8.2)	–8.4	–1.5(+2.3,–5.3)	–6.1
One year	–6.3(–0.8,–11.9)[c]	–15.7	–1.9(–0.0,–5.7)	–5.4
Two years	–14.2(–8.6,–19.7)[c]	–29.0	–6.0(–2.2,–9.8)[c]	–17.5
Three years	–27.4(–21.8,–33.0)[c]	–53.3	–12.4(–8.5,–16.2)[c]	–25.1

[a]The effect of temperature was significant for the one-, two-, and three-year results.
[b]Assay kit lot numbers used at each time point and number of months between analysis date and expiration date of kit given in parentheses: baseline (10 mon), one (10 mon), and four weeks (9 mon), NK2681; three months (5 mon), NM1081; six months (11 mon), PC2681; one year (9 mon; 7 mon), PG1681 (–20°C), PK2181 (–70°C); two years (15 mon), QK0181 and three years (1 mon), 104242.
[c]Significantly different from baseline values at $P < 0.05$.
[d]Confidence intervals not computed for three months time point (see text).

age emphasizes short-term studies (<6 mon), and the conclusions are variable. The results are influenced by the storage conditions employed and by the method used to quantitate Lp(a).

At 4°C, Sgoutas and Tuten (33) in a study involving serum samples observed a significant decrease of 30% (ELISA, Standard Diagnostics) and 12% (immunourbidimetric) in Lp(a) values after 16 d at 4°C. Cazzolato *et al.* (34) also using serum but with EDTA and NaN_3 added noted that Lp(a) concentrations were stable after 2 mon if measured by nephelometry but, if analyzed by Laurell electrophoresis the areas increased after 2 mon. In contrast, four studies storing plasma for up to 10 wk at 4°C noted no significant change in Lp(a) values. Albers and Hazzard (35) employing radial immunodifusion (RID) and Albers *et al.* (36) using a radioimmunoassay method noted that Lp(a) levels in EDTA plasma samples containing NaN_3 were unaffected by four and ten weeks of storage, respectively. Similarly, Fless *et al.* (31) and Taddei-Peters *et al.* (30), both analyzing filter sterilized EDTA plasma by an ELISA, reported no change in Lp(a) levels after one week and one month, respectively.

At –20°C, three studies report that Lp(a) in serum is stable during storage. Jauhiainen *et al.* (26) employing an immunoradiometric (IRMA) method reported that the serum concentration of a population with Lp(a) levels of over 20 mg/dL was unaffected by storage for 8.5 yr, but their results indicated considerable fluctuation among the values for individual samples with time. Similarly, Gaubatz *et al.* (37) using an electroimmunoassay reported that Lp(a) was stable for one year in serum samples. Cazzolato *et al.* (34) analyzing serum samples fortified with EDTA and NaN_3 noted no change in

Lp(a) concentration after 6 mon of storage. Both electrophoresis and nephelometry gave similar results although the authors emphasize that an initial freeze/thaw causes a 10 to 12% decline in Lp(a) concentrations. In contrast to these results, Craig *et al.* (38) using RID noted that Lp(a) concentrations in serum declined by 46% within 6 mon. Our ELISA results indicate that serum Lp(a) values diminish during storage at −20°C, but the decreases (arithmetic mean): 9.2% at 6 mon and 53.3% at 3 yr are at a slower rate than that noted by Craig *et al.* (38). This discrepancy is magnified if we compare our results for samples with over 8 mg/dL of Lp(a) to the Craig *et al.* (38) data who also used the 8 mg/dL cut off point. As both studies used serum samples with no additives, the clearest distinction between the two reports is that this investigation employed an ELISA (Standard Diagnostics) and Craig *et al.* (38) used RID to quantitate Lp(a). However, our rates of Lp(a) loss are similar to those noted by Kronenberg *et al.* (39) who studied plasma (EDTA) samples. Using an ELISA (Boehringer), they observed a 13% decline in Lp(a) concentration after two years. Albers and Hazzard (35) using RID noted that four weeks of storage had no effect on Lp(a) levels in plasma (EDTA, NaN_3) samples. In contrast, Usher *et al.* (29) also analyzing plasma (EDTA, NaN_3, Trasylol) samples and using two ELISA procedures (Standard Diagnostic, Boehringer) observed an apparent elevation of 8% in Lp(a) values after storage for four to six weeks at −20°C.

At −70°C, Craig *et al.* (38) (serum) reported that losses at −70°C were not significantly different from the extensive decreases observed at −20°C (see above). Our −70°C data (arithmetic mean) noted a 17.1 and 26.2% decline in Lp(a) values after two and three years' storage, respectively. Again our data for loss of Lp(a) are closer to those reported by Kronenberg *et al.* (39) for plasma (EDTA) samples: 7% after two years at −80°C as measured by ELISA (Boehringer). Fless *et al.* (31) and Taddei-Peters *et al.* (30) both studying filter sterilized EDTA plasma and using ELISA employing apo(a) capture and apoB detection antibodies reported that Lp(a) was stable for at least one month and one year, respectively.

The results for Lp(a) storage documented above are highly variable. This partly reflects the different storage conditions and analytical techniques utilized. As expected, losses are more apparent in serum than in plasma samples. The wide number of techniques involved makes comparison of the various methodologies difficult. However, as noted above, Sgoutas *et al.* (33) analyzing serum samples noted that an ELISA (Standard Diagnostics) is more sensitive to apparent losses than nephelometry. The high losses observed by Craig *et al.* (38) were obtained using RID.

Apparently discrepant results may also reflect differences in isoform frequency between the populations studied. Consequently, we analyzed the apo(a) phenotypes of our stored samples and results are available for 22 specimens. The three samples that gave inconclusive results contained low concentrations of Lp(a): 1.0, 1.1, and 3.7 mg/dL. Using apo(a) isoform 15 as the cutoff point, the population could be divided into three groups: both isoforms ($n = 7$) of small size (low); both isoforms ($n = 9$) of large size (high) and a third group ($n = 6$) with a combination of small and large isoforms (medium). We made the assumption that all single-banded samples (nine) were homozygous for the product detected, i.e., no null gene present. Statistical analysis indicated that at −20°C the effect of isoform size was of marginal significance ($P = 0.064$) but at −70°C, when the apparent loss is much less, the effect of isoform size was significant ($P = 0.024$). If only the high and low groups were compared, then the result at −20°C was similar but at −70°C the P value was now <0.005. At both temperatures the percentage decrease in Lp(a) concentration after storage for three years was greatest for the high molecular weight group. After stratifying the apo(a) isoforms based on size, the groups become small but the data suggest that stability of Lp(a) during storage is influenced by isoform size.

Inspection of our results (Table 1) indicates a strong linear component to the decreases in Lp(a) concentration, emphasizing the effect of storage time, and minimizing the influence of the time periods between collection and freezing and between thawing and analysis. However, the values obtained at three months are an exception. At this timepoint the Lp(a) concentrations had apparently increased (arithmetic mean) by 18.7 and 9.0% at −20°C and −70°C, respectively. It is possible for lipoprotein levels to ostensibly increase during storage as was noted for Lp(a) by Usher *et al.* (29). The epitope of the Lp(a) may change resulting in enhanced interaction with antibodies. It is questionable, however, whether this effect would only be manifested at the three-month measurement. Evaporation of samples may also cause an apparent increase in Lp(a) concentration, and care was taken to avoid this problem. It should also be noted that the −20 and −70°C samples were handled as separate batches; although both groups exhibited an apparent rise in Lp(a) concentration at three months, this was not observed at any other time point. An alternative explanation notes that in addition to any effects of storage, superimposed on the analyses is an analytical variation. Both the −20 and −70°C samples at three months were assayed using the same lot number kit (NM 1081), and the other time points involved several different lot numbers (Table 1).

Kronenberg *et al.* (39) have reported that degradation of Lp(a) at −20 or −80°C occurs predominantly during the first month of storage, but our studies failed to confirm this observation. Kronenberg *et al.* (39) also noted loss of Lp(a) in samples stored at −20 or −80°C was associated with one or three freeze/thaw cycles. Similarly, Sgoutas and Tuten (33) noted loss of Lp(a) in samples stored at −20°C after one (ELISA) or two (ITA) freeze/thaw cycles. If the samples were stored at −80°C, then loss of Lp(a) was noted after two (ELISA) or three (ITA) freeze/thaw cycles. We do not have information on one or two freeze/thaw cycles, but we have data indicating that the values for Lp(a) are not affected by three to eight freeze/thaw procedures prior to analysis (40).

Although it must be emphasized that our results are specific for the storage conditions used and the analytical proce-

dures employed, the apparent decline in Lp(a) during storage stresses the need to minimize potential losses. Specific procedures employed must bear in mind the analyses that are subsequently to be performed, but several precautions can be considered. It is known that collection of samples into EDTA reduces degradation resulting from autoxidation and enzymatic activity by chelating heavy metal ions (41). Correction must then be made for the osmotic effect of EDTA which causes an artificial fall in lipoprotein concentration (42). In addition, protease inhibitors such as aprotinin may be added to the samples to minimize proteolytic activity arising from native or bacterial enzymes (43). Finally, an antibacterial agent such as Thimerosal can be included (44).

In conclusion, our results indicate that after storage of serum samples at −20 and −70°C for three years the values observed for Lp(a) are decreased (geometric mean) by 36.9 and 19.1% and (arithmetic mean) by 53.5 and 26.2%, respectively. Nevertheless, the concentrations of Lp(a) in stockpiled samples remain closely correlated with baseline values indicating that although absolute values change, stored samples can still be used for comparisons. However, the duration the aliquots have been stored at −70°C should not vary more than six months between samples. In addition, samples stored at −70°C for three or more years should be matched for apo(a) isoform size.

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COMMUNICATION

The Sterols of *Cucurbita moschata* ("calabacita") Seed Oil

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ABSTRACT: From the sterol fraction of seed oil from commercial *Cucurbita moschata* Dutch ("calabacita") Δ^5 and Δ^7 sterols having saturated and unsaturated side chain were isolated by chromatographic procedures and characterized by spectroscopic (^{1}H and ^{13}C-nuclear magnetic resonance, mass spectrometry) methods. The main components were identified as 24*S*-ethyl 5α-cholesta-7,22*E*-dien-3β-ol (α-spinasterol); 24*S*-ethyl 5α-cholesta-7,22*E*,25-trien-3β-ol (25-dehydrochondrillasterol); 24*S*-ethyl 5α-cholesta-7,25-dien-3β-ol; 24*R*-ethyl-cholesta-7-en-3β-ol (Δ^7-stigmastenol) and 24-ethyl-cholesta-7,24(28)-dien-3β-ol ($\Delta^{7,24(28)}$-stigmastadienol).

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In previous works the general chemical composition of the fruit pulp, the acidic composition of crude seed oil, and the chemical composition of the seed defatted meal flour from *Cucurbita moschata* Dutch ("calabacita") (1) were evaluated (2–4). Continuing with the study of the lipids from the same seed oil, the sterols were isolated from the unsaponifiable fraction and analyzed by chromatographic and spectroscopic methods. We report now the sterol composition, including the absolute configuration at C-24 of the major components, of the natural occurring Δ^5 and Δ^7 *C. moschata* sterols. In contrast to previous studies finding epimeric mixtures at C-24 (5–7), we detected single stereoisomers.

MATERIALS AND METHODS

Cucurbita moschata Dutch was harvested at Buenos Aires and Mendoza provinces, Argentina. Ground whole seeds (715 g) were extracted with hexane (Soxhlet) yielding 222 g of crude oil (33.2% on dry basis). Saponification of 14 g of oil afforded unsaponificable fraction (250 mg) from which the sterol fraction was obtained by preparative thin-layer chromatography. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded in a Bruker AC-200 (Karlsruhe, Germany) and in a Bruker AM-500 spectrometers. Chemical shifts are listed in δ (ppm) relative to tetramethylsilane employed as internal standard. Coupling constants (*J*) are presented in Hz. ^{13}C NMR spectra were full decoupled. Gaschromatography–mass spectrometry (GC–MS) analyses were performed on a VG TRIO-2 (Manchester, United Kingdom) instrument. High-resolution mass spectra (HRMS) were registered in a VG-ZAB BEQ spectrometer. Preparative high-performance liquid chromatography (HPLC) was carried out in a Micromeritics chromatograph (Norcross, GA) with a solvent delivery system model 750, a Thermo Separation Products refractive index detector model refractoMonitor® IV (Fremont, CA), and a Beckman Ultrasphere ODS-2 column (250 × 10 mm, 5 μm) (Fullerton, CA) eluting with absolute methanol at a flow rate of 3.0 mL/min.

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Abbreviations: GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; NMR, nuclear magnetic resonance.

RESULTS AND DISCUSSION

The unsaponificable fraction from a hexane extract of seed oil from *C. moschata* Dutch was separated by preparative thin layer chromatography (8). The sterol fraction was isolated and analyzed by GC and GC–MS methods. These analyses were conducted on free sterols as well as on their trimethylsilyl ether and acetyl derivatives. The mixture contained Δ^5 and Δ^7 derivatives with saturated and unsaturated side chains. The mass spectra of the derivatives allowed unequivocal assignments of Δ^5 and Δ^7 sterols. The latter were characterized by the presence of molecular ions for acetates and the absence of M-129 and 129 fragments for the silylated derivatives (data not shown) (9).

Table 1 shows the 11 components detected in the sterol fraction together with their percentage in the mixture, GC–relative retention time data and the main fragments of their respective mass spectrum registered for the free sterols and for the respective acetylated derivatives.

The sterols were also separated by preparative HPLC, and the major members corresponding to the single peaks were further analyzed by NMR spectrometry and MS. The ^{1}H NMR analysis also indicates the presence of a Δ^7 double bond according to the chemical shift of angular methyls, 0.54 for H-18 and 0.80 ppm for H-19 (10,11) and to the chemical shift of the vinyl proton (H-7) at 5.16 ppm. The presence of a Δ^7 series was clearly confirmed by ^{13}C NMR spectra in which signals corresponding to C-7 and C-8 are observed at around 117 and 139 ppm, respectively (12–14).

24S-Ethyl 5α-cholesta-7,22E-dien-3β-ol (α-spinasterol) (**7**). HPLC retention time 26.7 min. HRMS: Calc'd 412.3705.

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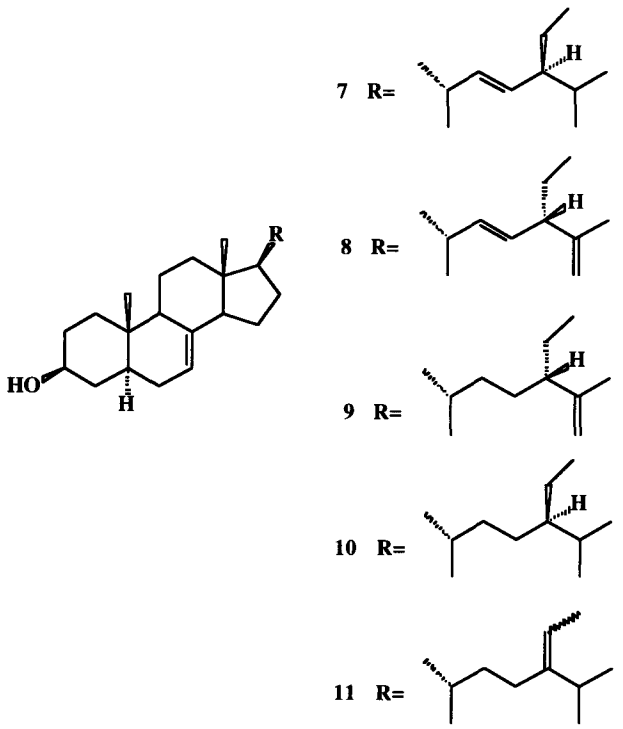
TABLE 1
Composition, Relative Retention Time (RRT),[a] and Mass Spectra of Sterols from *Cucurbita moschata* Seed Oil

Sterol	Sterol systematic nomenclature	RRt	%	Mass spectrum free sterols	Mass spectrum acetyl derivatives
1	Cholest-5-en 3β-ol	1.00	0.8	386 (M, 96), 371 (54), 368 (72), 353 (47), 301 (65), 275 (100)	368 (M-60,100), 353 (25), 313 (18), 260 (18), 247 (15), 213 (16)
2	24ξ-Methyl-cholesta-5,22-dien-3β-ol	1.12	3.2	398 (M, 28), 380 (8), 365 (12), 314 (16), 299 (22), 271 (37), 213 (45), 159 (56), 145 (74), 107 (98), 95 (100)	380 (M-60, 100), 365 (13), 313 (6), 255 (13), 253 (13), 213 (19)
3	24ξ-Methyl-cholest-5-en-3β-ol	1.13	4.7	400 (M, 15), 382 (11), 367 (10), 315 (26), 289 (17), 273 (21), 255 (33), 213 (39), 145 (66), 107 (100)	382 (M-60, 100), 367 (17), 274 (12), 255 (23), 253 (8), 213 (21)
4	24ξ-Methyl-5α-cholesta-3β-ol	1.15	0.7	402 (M, 100), 387 (6), 369 (4), 275 (10), 257 (31), 233 (43), 217 (18), 215 (36)	444 (M, 7), 384 (19), 369 (9), 275 (12), 257 (5), 255 (12), 215 (57), 43 (100)
5	24ξ-Ethyl-cholesta-5,22-dien-3β-ol	1.17	4.10	412 (M, 13), 379 (3), 351 (8), 300 (13), 275 (35), 255 (39), 213 (24), 159 (22), 83 (98), 55 (100)	394 (M-60, 100), 379 (5), 351 (9), 255 (53), 253 (18), 213 (21)
6	24ξ-Methyl cholest-7-en-3β-ol	1.22	4.80	400 (M, 37), 385 (17), 367 (4), 273 (23), 271 (24), 255 (100), 231 (35), 213 (50)	442 (M, 51), 427 (13), 367 (8), 315 (12), 313 (16), 255 (100), 213 (38)
7	24*S*-Ethyl 5α-cholesta-7,22*E*-dien-3β-ol	1.29	33.5	412 (M, 18), 397 (13), 300 (15), 271 (100), 255 (65), 246 (22), 231 (24)	454 (M, 15), 439 (5), 394 (18), 313 (31), 271 (100), 255 (49), 213 (23)
8	24*S*-Ethyl 5α-cholesta-7,22*E*,25-trien-3β-ol	1.30	10.4	410 (M, 9), 381 (4), 300 (11), 271 (100), 255 (43), 213 (26), 137 (41), 107 (67)	452 (M, 10), 437 (15), 392 (5), 313 (32), 271 (100), 255 (56), 213 (38)
9	24*S*-Ethyl 5α-cholesta-7,25-dien-3β-ol	1.37	19.3	412 (M, 7), 397 (12), 379 (4), 271 (100), 255 (37), 213 (28), 173 (18), 161 (24)	454 (M, 10), 439 (13), 394 (3), 313 (100), 288 (5), 253 (7), 227 (13), 213 (15)
10	24*R*-Ethyl-cholest-7-en-3β-ol	1.39	9.6	414 (M, 30), 399 (20), 381 (9), 273 (23), 255 (100)	456 (M, 72), 441 (18), 315 (11), 255 (100), 213 (34)
11	24-Ethyl-cholesta-7,24(28)-dien-3β-ol	1.43	8.4	412 (M, 2), 314 (2), 271 (100), 253 (19), 253 (9), 213 (14), 173 (9)	454 (M, 2), 439 (2), 356 (33), 313 (100), 253 (9), 213 (10)

[a]RRt, relative to cholesterol.

Found 412.3705. ^{1}H NMR (Cl_3CD, 500 MHz): δ 0.553 (*s*, 3 H, H-18), 0.801 (*s*, 3H, H-19), 0.84 (*t*, *J* = 6.4, 3 H, H-29), 1.05 (*d*, *J* = 6.6, 3 H, H-21), 3.60 (*m*, 1 H, H-3), 5.10 (*dd*, *J* = 15.2, 8.7, 1 H, H-23), 5.15 (*m*, 1 H, H-7), 5.16 (*dd*, *J* = 15.2, 8.7, 1 H, H-22). ^{13}C NMR (Cl_3CD) δ 12.07 (C-18), 12.23 (C-29), 13.04 (C-19), 19.01 (C-27), 21.08 (C-21), 21.37 (C-26), 21.58 (C-11), 23.04 (C-15), 25.38 (C-28), 28.49 (C-16), 29.68 (C-6), 31.53 (C-2), 31.88 (C-25), 34.26 (C-10), 37.19 (C-1), 38.06 (C-4), 39.51 (C-12), 40.32 (C-5), 40.77 (C-5), 43.32 (C-13), 49.51 (C-9), 51.26 (C-24), 55.16 (C-14), 56.00 (C-17), 71.08 (C-3), 117.48 (C-7), 129.52 (C-23), 138.15 (C-22), 139.58 (C-8).

*24S-Ethyl 5α-cholesta-7,22E,25-trien-3β-ol (25-dehydrochondrillasterol) (***8***).* HPLC retention time 20.0 min. HRMS: Calc'd 410.3549. Found 410.3546. ^{1}H NMR (Cl_3CD, 500 MHz): δ 0.548 (*s*, 3 H, H-18), 0.800 (*s*, 3 H, H-19), 0.838 (*t*, *J* = 7.3, 3 H, H-29), 1.02 (*d*, *J* = 6.6, 3 H, H-21), 1.651 (*s*, 3 H, H-26), 3.60 (*m*, 1 H, H-3), 4.698 (*m*, 2 H, H-27), 5.161 (*m*, 1 H, H-7), 5.19 (*dd*, *J* = 15.2 ,8.2, 1 H, H-22), 5.25 (*dd*, 15.2, 8.2, 1 H, H-23). ^{13}C NMR (Cl_3CD): δ 12.07 (C-18 and C-29), 13.04 (C-19), 20.94 (C-26), 21.56 (C-11), 22.98 (C-15), 25.74 (C-28), 28.27 (C-16), 29.68 (C-2 and C-6), 31.53 (C-2), 32.42 (C-4), 34.25 (C-10), 37.19 (C-1), 38.05 (C-5), 39.50 (C-12), 40.33 (C-20), 43.37 (C-13), 49.51 (C-9), 52.03 (C-24), 55.14



SCHEME 1

(C-14), 56.89 (C-17), 71.07 (C-3), 109.56 (C-26), 117.52 (C-7), 130.25 (C-23), 137.03 (C-22), 139.53 (C-8).

24S-Ethyl-cholesta-7,25-dien-3β-ol (**9**). HPLC retention time 23.3 min. HRMS: Calc'd 412.3705. Found 412.3807. ^{1}H NMR (Cl_3CD): δ 0.53 (*s*, 3 H, H-18), 0.79 (*s*, 3 H, H-19), 0.80 (*t*, *J* = 7.3, 3 H, H-29), 0.91 (*d*, *J* = 6.3, 3 H, H-21), 1.57 (*s*, 3 H, H-27), 3.59 (*m*, 1 H, H-3), 4.65 (*m*, 1 H, H-26A), 4.73 (*m*, 1 H, H-26B), 5.15 (*m*, 1 H, H-7). ^{13}C NMR (Cl_3CD): δ 11.82 (C-29), 12.02 (C-18), 13.04 (C-19), 18.78 (C-21 & C-26), 21.58 (C-11), 22.96 (C-15), 26.54 (C-28), 27.88 (C-16), 29.55 (C-23), 29.68 (C-6), 31.53 (C-2), 33.66 (C-22), 34.24 (C-10), 35.99 (C-20), 37.18 (C-1), 38.05 (C-4), 39.61 (C-5), 40.30 (C-12), 43.42 (C-13), 49.53 (C-9 & C-24), 55.08 (C-14), 56.16 (C-13), 71.08 (C-3), 111.33 (C-27), 117.43 (C-7), 139.61 (C-8), 147.59 (C-25).

24R-Ethyl-cholesta-7-en-3β-ol (**10**). HPLC retention time 29.9. HRMS: Calc'd 414.3862. Found 414.3862. ^{1}H NMR (Cl_3CD): δ 0.54 (*s*, 3 H, H-18), 0.80 (*s*, 3 H, H-19), 0.928 (*d*, *J* = 6.1, 3 H, H-21), 3.60 (*m*, 1 H, H-3), 5.17 (*m*, 1 H, H-7).

24-Ethyl-cholesta-7,24(28)-dien-3β-ol (**11**). HPLC retention time 24.6. HRMS: Calc'd 412.3705. Found 412.3795. ^{1}H NMR (Cl_3CD): δ 0.54 (*s*, 3 H, H-18), 0.80 (*s*, 3 H, H-19), 0.98 (*d*, *J* = 6.9, 6 H, H-26 and H-27), 3.60 (*m*, 1 H, H-3), 5.08 (*m*, 1 H, H-3), 5.08 (*m*, 1 H, H-28), 5.16 (m, 1 H, H-7).

The most abundant components, all belonging to the Δ^7 series, were 24*S*-ethyl 5α-cholesta-7,22E-dien-3β-ol (spinasterol, the 24α-epimer) (**7**) being the principal one accounting for almost 34% of the total mixture, 24*S*-ethyl 5α cholesta-7,22*E*,25-trien-3β-ol (25-dehydro condrillasterol, the 24β-epimer) (**8**) (10%) and 24*S*-ethyl-cholesta-7,25-dien-3β-ol (24β-epimer) (**9**) (19%). Δ^7-Stigmastenol (24α-epimer) (**10**) (10%) and $\Delta^{7,24(28)}$-stigmastadienol (**11**) (8%) were also present as important components.

It is interesting to point out that previous works dealing with 24-ethyl-Δ^7-sterols indicated co-occurrence of carbon-24 epimeric mixtures although with predominance of 24-α epimers (5–7). In the present case, we undoubtedly detected C-24 single stereoisomers. This assertion is based on the careful analysis of the NMR spectra and chromatographic behavior of the purified compounds.

The absolute configuration at C-24 were assigned by comparison of published NMR data from sterols of other species of the Cucurbitacea family (6–7). The structures of these sterols are presented in Scheme 1.

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ERRATUM

Due to a printer error in the September issue of *Lipids*, the photographs on pages 895 and 985 were transposed.

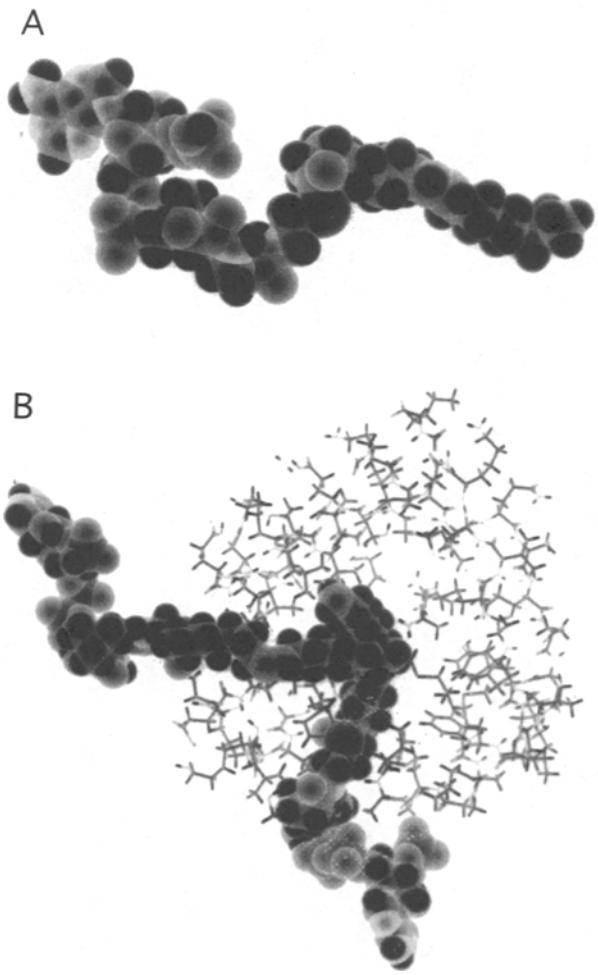


FIG. 1. Structure of free (panel A) and liver fatty acid binding protein bound (panel B) oleoyl CoA. These structures were prepared by Silicon Graphics (Mountain View, CA) computer modeling as described elsewhere (Ref. 127).

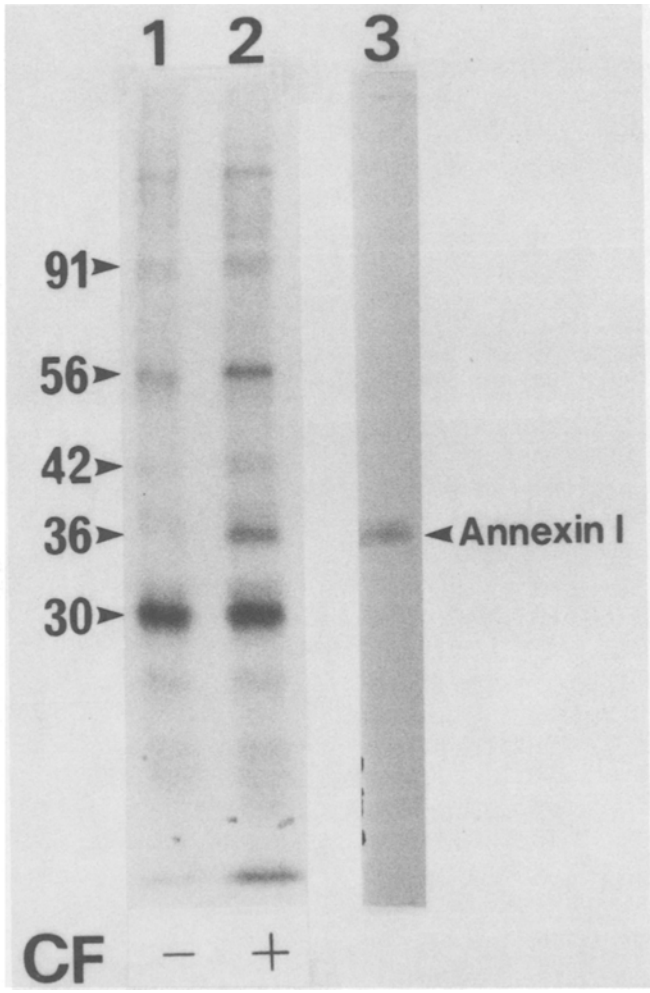


FIG. 3. Western blot analysis of the 36 kDa particulate protein using anti-annexin I. Lanes 1–2, autoradiography; lane 3, the blotting: CF, the protein kinase C cofactors (5 μg/mL 1-oleoyl-2-acetyl-*sn*-glycerol, 25 μg/mL phosphatidylserine, and 1 μM Ca^{2+}).

Epoxy Allylic Carbocations as Conceptual Intermediates in the Biogenesis of Diverse Marine Oxylipins

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ABSTRACT: Marine organisms, especially marine algae, are extremely rich in a diversity of novel oxylipin structures. Many of these oxylipins contain functionalities and rings of a type and location unknown in mammalian systems. In this perspective reviewing marine oxylipins, a proposal is formulated for the central intermediacy of an epoxy allylic carbocation in the biogenesis of these diverse structures. This proposal is strengthened by the relatively large number of examples which are consistent with this type of mechanistic transformation.
Lipids 31, 1215–1231 (1996).

In the last 20 years, virtually all classes of marine organisms have yielded unusual oxylipin structures, collectively identifying marine creatures as the richest source for structural diversity in this chemical class (1). Structural motifs particularly noteworthy among marine oxylipins, because of their abundance and structural uniqueness, include unusual positions of oxidation (1,2) incorporation of halogen atoms (3,4), and carbocycles involving positions not usually involved in ring formation in mammalian prostaglandins (5). Coincident with the unusual structures of these metabolites is a range of intriguing biological properties, some with medical application (e.g., antiproliferative) (6) and some with profound ecological roles (4,7). However, appreciation of the chemistry, biochemistry, and ecology of marine oxylipin production is still very much in its infancy, and there are many more cases wherein these features remain concealed than known.

In formulating this review, I have considered this surprisingly diverse array of marine oxylipins, many of which my laboratory has discovered over the past 10 years, with an aim of uncovering potential points of commonality in their structure and biogenesis (Fig. 1). In this regard, I hoped to find features which would make these structures, individually as well as collectively, more comprehensible in terms of their chemistry and biochemistry, and perhaps even their evolutionary origins.

Abbreviations: 2D, two-dimensional; CD, circular dichroism; EAC, epoxy allylic carbocation; GC, gas chromatography; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HODTA, hydroxyoctadecatetraenoic acid; HOTE, hydroxyoctadecatrienoic acid; HpEPE, hydroperoxyeicosapentaenoic acid; HpETE, hydroperoxyeicosatetraenoic acid; HpODTA, hydroperoxyoctadecatetraenoic acid; LO, lipoxygenase; MC, (−)-menthoxycarbonyl; MS, mass spectrometry; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

Marine organisms are characteristically rich in polyunsaturated fatty acids, particularly those of the ω3 class (8). Many of these fatty acids are substrates for diverse lipoxygenase enzymes which stereospecifically oxidize these to diene-hydroperoxides. Fatty acids of various chain lengths (e.g., C_{18}, C_{20}, and C_{22}) are often acceptable substrates for a particular oxidation pathway and give rise to homologous series of oxylipins (9–11). As a group, marine organisms are also rich in the diversity of sites in the carbon chain to which lipoxygenases (LO) show specificity. [The convention followed in this review is that the positional specificity of lipoxygenases acting on C_{20}-derived fatty acids is identified relative to the carboxyl terminus (e.g., 5-lipoxygenase, 8-lipoxygenase), whereas their specificity on C_{18} acids is identified relative to the methyl terminus (e.g., ω6 lipoxygenase).] Marine oxylipins which likely derive from action of 5-, 8-, 9-, 11-, 12-, 15- (1,2), or "ω3" lipoxygenases (12) have been reported (1,2). With only a few exceptions (13–15), mechanistic and enzymological features of this wealth of marine LO have gone unstudied. Nevertheless, either through direct product isolation or inference, the same general mechanism for conversion of a 1,4-diene to a conjugated diene hydroperoxide is postulated and seems reasonable (16). This review's attempt to identify unifying features of marine oxylipin biogenesis focuses on steps subsequent to the LO-initiated metabolism of endogenous polyenoic fatty acids to hydroperoxides, as this initiating step has already been identified as being in common to many marine oxylipins (2). The ensuing steps involve reaction and rearrangement chemistry of this hydroperoxide.

For example, tissues and life stages of various higher plants, especially the seeds and seedlings of cereals, as well as lower plants and some animals such as coral, contain an enzymatic activity which catalyzes the dehydration of the LO-derived hydroperoxides to allene oxides (17). This activity is due to allene oxide synthase (hydroperoxide dehydrase), a membrane-bound enzyme that in plants is associated with chloroplasts (18). It was purified to homogeneity from flaxseed and characterized as a 55 kD hemoprotein of the cytochrome P-450 class (19). Recent insight into the mechanism of allene oxide formation (Fig. 2) was obtained through fortuitous isolation of a series of epoxyalcohols when an alternative substrate was provided to the enzyme (20). Consistent with the mechanism and activity of cytochrome P-450, the

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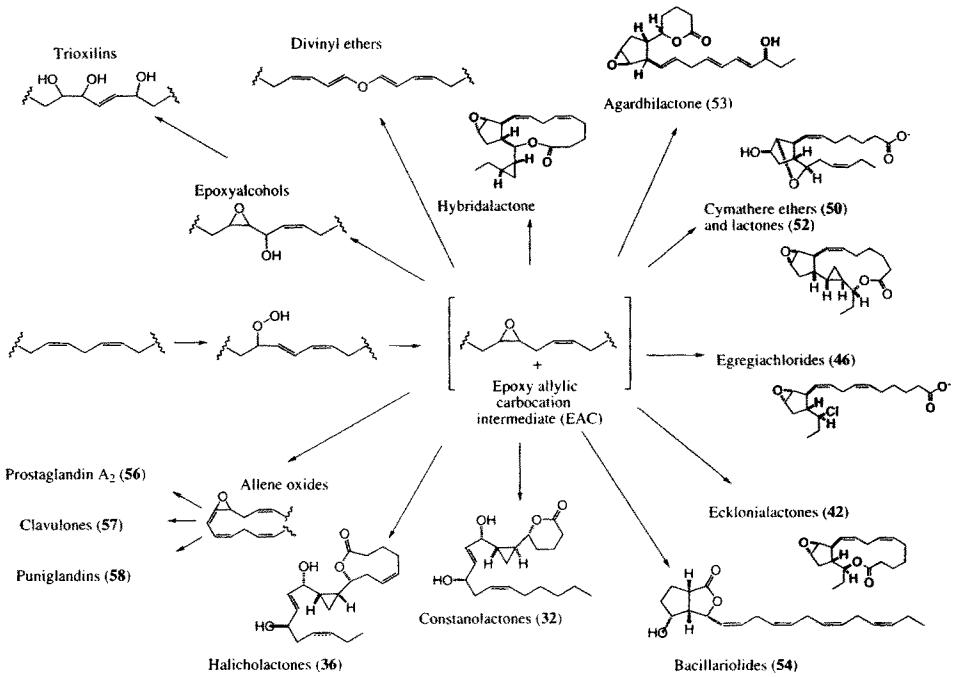


FIG. 1. Overview of the proposed central intermediacy of epoxy allylic cations (EAC) in the biogenesis of diverse marine oxylipins.

hydroperoxide is proposed to be homolytically cleaved, and the resulting allylic alkoxyl radical then cyclizes to an epoxide, thus forming a carbon-centered radical. Under normal processing by the enzyme, and in analogy to mechanisms worked out for the prostacyclin and thromboxane synthases (21), a one electron oxidation by the iron-oxo complex leads to an epoxy allylic carbocation (EAC) which upon proton loss gives an allene oxide, the precursor to phytodienoic and jasmonic acids in higher plants (22), and presumably, diverse prostaglandin structures in marine corals (Fig. 2) (23,24).

It is the contention of this review that all of the steps in this mechanism, except the last, are in operation in the formation of a diversity of marine oxylipins, as an EAC is in fact an ideal conceptual intermediate in the biogenesis of diverse marine oxylipin structures (Fig. 1). Such an intermediate has been previously proposed in the biosynthetic pathway for

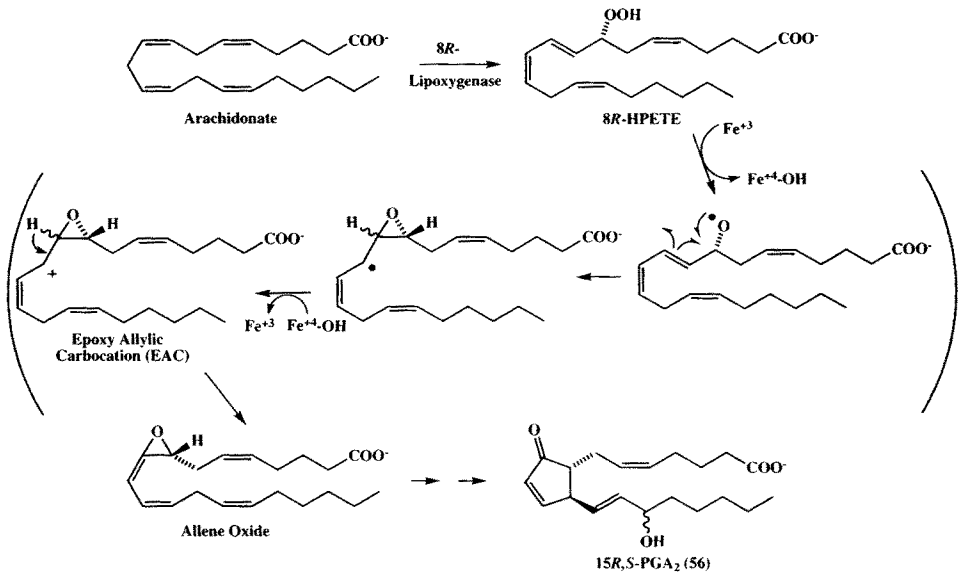


FIG. 2. Allene oxide biosynthesis involving an intermediate alkoxyl radical and epoxy allylic cation (adapted with modifications from Ref. 20). In marine corals such as *Plexaura homomalla,* this biosynthetic pathway has been postulated to lead to PGA_2 (**56**) production.

epoxyalcohols (25), α- and γ-ketols (26), allene oxides (20,27,28), divinyl ethers (29), and a number of marine-derived carbocyclic oxylipins (30–32). This proposal not only gives potential insight into the mechanism of marine oxylipin formation, but also into the enzymology and evolutionary relationship of this biosynthetic process in diverse marine creatures. Finally, while it is beyond the scope of this review to describe alternative hypotheses for each oxylipin metabolite, it should be acknowledged that for many discussed herein, alternative biogeneses can be proposed that are mechanistically and biochemically rational. It is precisely this uncertain origin which makes their structures so fascinating, and points out the necessity for biosynthetic experimentation in this area.

CENTRALITY OF EPOXY ALLYLIC CARBOCATIONS IN THE BIOGENESIS OF DIVERSE OXYLIPINS

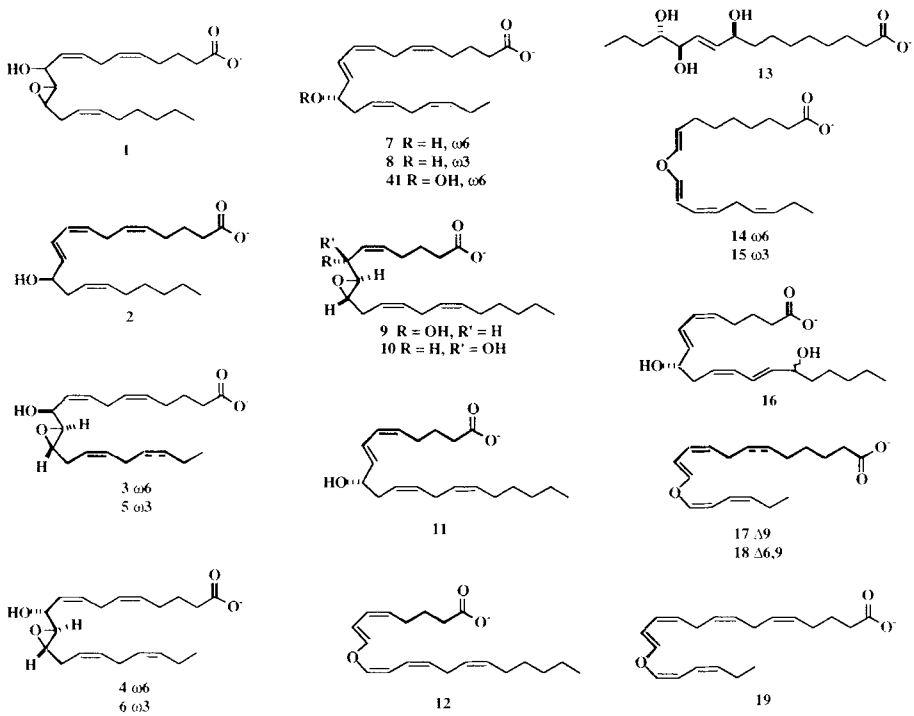
Those marine oxylipins which potentially arise through a hydroperoxide-epoxy allylic carbocation manifold are arranged in the following sections, first into acyclic products and then into carbocyclic products. This latter section is further subdivided into cyclopropyl and cyclopentyl oxylipins. Collectively, this large number of metabolites (greater than 50) which is envisioned to arise *via* an EAC intermediate provides an abundance of support to the idea that this is a general metabolic feature of oxylipin formation in marine organisms. In the ensuing sections, the structure and methods of structural analysis are first presented, and are then followed in each case by biogenetic hypotheses.

OXYLIPINS WITHOUT CARBOCYCLES

1. Epoxyalcohols

The occurrence of epoxyalcohols in the lipid extracts of marine organisms was first reported from the tropical red algae, *Platysiphonia miniata* and *Cottoniella filamentosa*, in 1990 (33). Hepoxilin B_3 (**1**) (see Scheme 1—compound structures 1–19,41) was found as a minor metabolite of both of these algae and co-occurred with the well recognized LO metabolite, 12-hydroxyeicosatetraenoic acid (12-HETE, **2**). From *P. miniata*, the hydroxyacid was characterized as the *S* stereoisomer (**7**). While the epoxide was shown to be *trans* in both isolates of hepoxilin B_3, in neither case were other features of the stereochemistry determined.

Subsequently, both the *erythro* and *threo* isomers of hepoxilin B_3 (**3**,**4**) and hepoxilin B_4 (**5**,**6**) were isolated from another tropical red alga, *Murrayella periclados* (34). The relative stereochemistry of these two pairs of C-10 epimers was deduced from consideration of their relative polarity on thin-layer chromatography (TLC) as well as the H10 ^{1}H nuclear magnetic resonance (NMR) chemical shift and H10-H11 coupling constant (35). Absolute stereochemistries in the *erythro* isomer of hepoxilin B_3 and the *threo* isomer of hepoxilin B_4 were determined by derivatization and cleavage to form the (−)-menthoxycarbonyl (MC) derivative of dimethyl 2*S*-hydroxyundecane-1,11-dioate (from *erythro* B_3, **3**) and the (−)MC derivative of dimethyl 2*R*-hydroxyundecane-1,11-dioate (from *threo* B_4, **6**)(35). These were compared with authentic standards and defined the algal metabolites as *erythro*-11*S*,12*S*-epoxy-10*S*-hydroxy-5*Z*,8*Z*,14*Z*-eicosatrienoic acid



SCHEME 1

(**3**) and *threo*-11*S*,12*S*-epoxy-10*R*-hydroxy-5*Z*,8*Z*,14*Z*,17*Z*-eicosatetraenoic acid (**6**), respectively. The co-occurrence of the 12-LO metabolites 12*S*-HETE (**7**) and 12*S*-HEPE (**8**) supports the contention that the hepoxilin derivatives derive in these algae from rearrangement of putative 12*S*-HpETE (**46**) or 12*S*-HpEPE precursors (36).

Two new epoxyalcohols also have been obtained from the lipid extract of the Oregon red alga *Polyneura latissima* (37). These structures are unique in that the epoxide is located at C_8-C_9 and the alcohol (of both stereochemistries) is at C_7 [8*S**,9*S**-epoxy-7*S**-hydroxy-5*Z*,11*Z*,14*Z*-eicosatrienoic acid (**9**) and 8*S**,9*S**-epoxy-7*R**-hydroxy-5*Z*,11*Z*,14*Z*-eicosatrienoic acid (**10**)]. As these co-occurred with 9*S*-HETE (**11**), it is reasonable to propose the activity of a 9-LO in this seaweed which results in a 9-hydroperoxy intermediate. This is then either reduced (**11**), rearranged to epimeric epoxyalcohols (**9** and **10**), or rearranged to a novel divinyl ether (**12**, see below).

As shown in Figure 3, all of the above epoxyalcohols could conceptually arise *via* an EAC intermediate which in turn would derive from a dienyl hydroperoxide. The resulting EAC could be trapped with water to form the corresponding epoxyalcohols. However, it has been proposed that 1,2-epoxides with a 3-hydroxy or 5-hydroxy group (the "1" position is defined as the site of initial hydroperoxidation) are formed *via* radical processes (hydroperoxide dehydrase) while only epoxyalcohols of the 1-hydroxy-2,3-epoxy or 1-hydroxy-4,5-epoxy are formed *via* ionic mechanisms (hydroperoxide isomerase) (17). In this case, it is possible that in algae and other marine organisms the carbon-centered radical formed en route to an EAC (Fig. 2) is quite often trapped through an "oxygen rebound-type" mechanism to form the abundance of epoxyalcohols described above.

2. Trioxilin from *Lyngbya majuscula*

The marine cyanobacterium (blue-green alga) *L. majuscula* is a source of the hepoxilin-type hydrolysis product 9*S*,12*R*,13*S*-trihydroxy-10*E*,15*Z*-octadecadienoic acid (**13**)(38), and hence, is related to the homologous trioxilin of mammalian occurrence (39). The C_{20} homologs of these triols have been isolated from various mammalian tissues and have been shown to derive from hepoxilin epoxide hydrolase opening of hepoxilin A_3 (Fig. 3) (40). However, these same metabolites have also been isolated as hepoxilin A_3 hydrolysis artifacts, a process promoted under acidic conditions (40). In this study of *L. majuscula* chemistry (38), a nonacidic extraction and workup were used which favor the triol metabolite as the true natural product. Inherent in this biogenetic proposal for the formation of **13** is the intermediacy of 12*S*,13*S*-epoxy-9*S*-hydroxy-10*E*,15*Z*-octadecadienoic acid, itself formed *via* the rearrangement of 13*S*-hydroperoxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid (Fig. 3). In turn, the hydroperoxide likely originates from action of an ω6-lipoxygenase (C_{13} oxygenation) on α-linolenic acid.

3. Divinyl Ethers from Red and Brown Algae

While divinyl ethers [e.g., colneleic (**14**) and colnelenic acids (**15**)] were first found as metabolites of a terrestrial plant

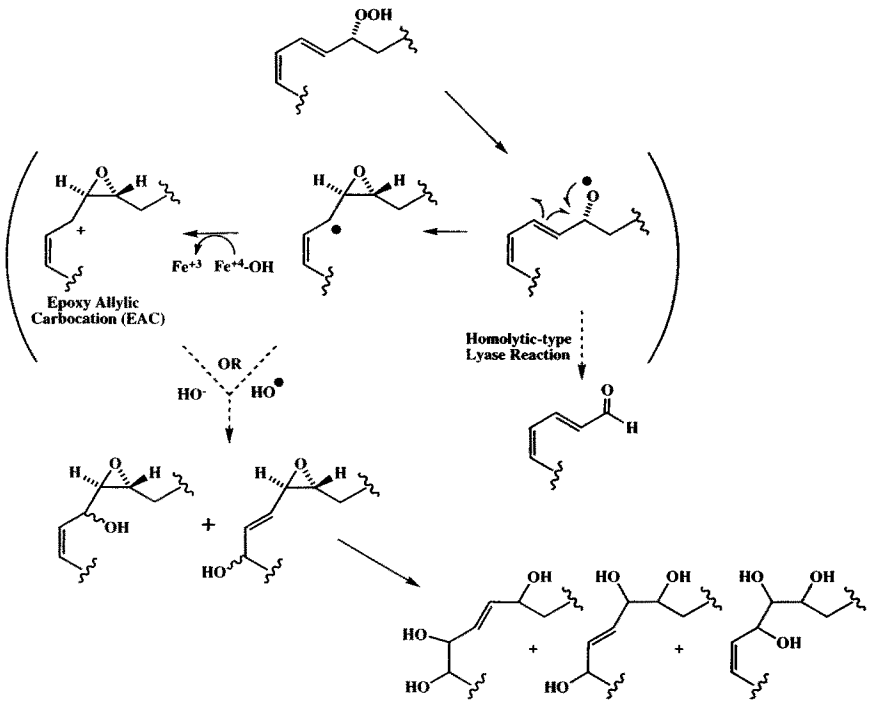


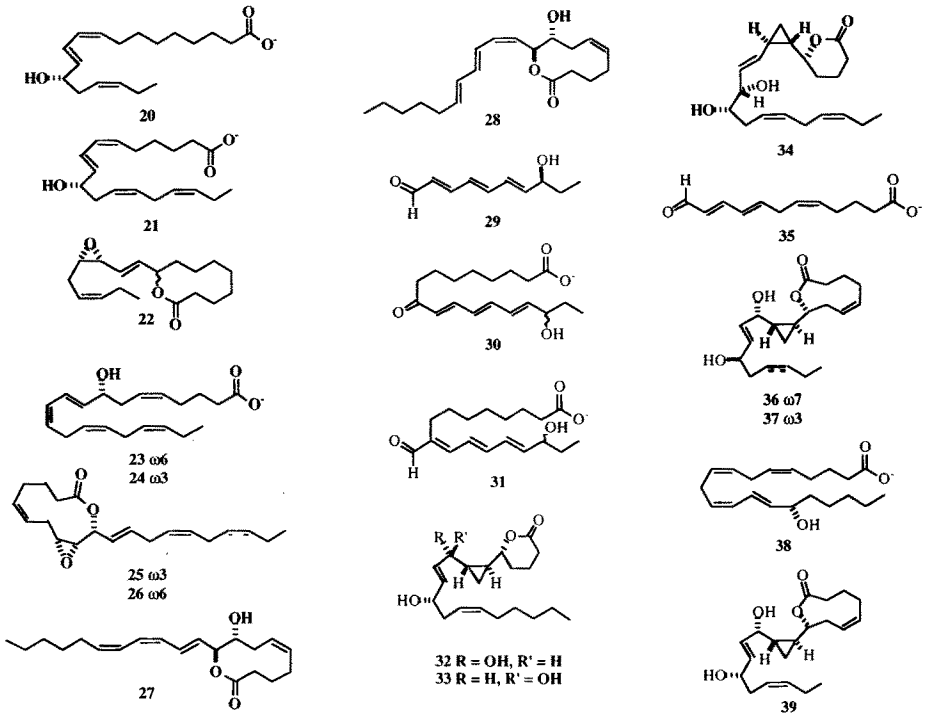
FIG. 3. Alternative radical and ionic pathways to epoxyalcohol (hepoxilins) and subsequent triol biosynthesis (trioxilins).

(potato)(41), their initial discovery from a marine organism was from the Californian red alga *Polyneura latissima* (37). The new compound, 8-[1′Z,3′Z,6′Z-dodecatrienyloxy]-5Z,7E-octadienoic acid (**12**), co-occurred with two epimeric epoxyalcohols (see above, **9** and **10**), a diol (**16**) and the simple hydroperoxide reduction product, 9*S*-HETE (**11**). Subsequently, divinyl ethers were also discovered from the brown alga *Laminaria sinclairii* (9). Collected from the Oregon coast, this kelp was rich in oxylipins and contained three new divinyl ethers (**17–19**) as well as five different monohydroxy polyunsaturated fatty acids. However, two other species of *Laminaria* were reported in the same work to contain only an assortment of the monohydroxy polyunsaturated fatty acids and none of the divinyl ethers.

The biogenesis of divinyl ethers in higher plants has been studied in considerable detail by Gallaird and Matthew (42), Crombie *et al.* (29), and Fahlstadius and Hamberg (43), and has been proposed to involve the rearrangement of an unsaturated hydroperoxide into an EAC of the type central to this review (29). Subsequently, biogenesis of divinyl ethers is envisioned to involve loss of a proton adjacent to the epoxide and electron shift from the epoxide C–C bond to quench the carbocation. In the case of *P. latissima*, this biogenetic pathway would again implicate a putative 9-LO, consistent with the origin of the co-occurring hydroxy acid and epoxyalcohols (35). In the case of *L. sinclairii*, a 15-lipoxygenase (or an ω6-lipoxygenase in the C_{18} compounds) is suggested, which in turn, is also consistent with the pattern of oxidation in the co-occurring hydroxy acids (9).

4. Carbon Chain-Cleaved Oxylipins from Various Algae

Products deriving from action of a putative "hydroperoxide lyase" have been obtained from a diversity of red, green, brown, and blue-green algae (1,2). Furthermore, in these life forms, most of the products observed are consistent with cleavage by the "homolytic-type" hydroperoxide lyase (i.e., products are formed by scission of the C–C bond between the hydroperoxide-bearing carbon and the α-methylene). In this respect, a hydroperoxide lyase was isolated and characterized from a freshwater cyanobacterium (*Oscillatoria* sp.) which converts 13*S*-hydroperoxyoctadecadienoic acid to 13-oxotrideca-9,11-dienoic acid (44). The "heterolytic-type" hydroperoxide lyase produces products arising from C–C scission between the hydroperoxide-bearing carbon and the α-olefinic carbon and is common in higher plants (17). Various mechanistic suggestions have been put forth to explain the formation of these cleavage products, some of which have implicated the intermediacy of an EAC (29). It should be noted that the terms "homolytic" and "heterolytic" represent terms to classify these two reactions and are not proven mechanistic definitions, but rather, remain proposals at the present time. Nevertheless, most microalgal and macroalgal lyase products are best rationalized as products of the "homolytic" process. It is conceivable that carbon-chain scission reactions in algae represent an alternative branch to the pathway herein proposed and emanate from the intermediate alkoxyl radical (Fig. 3); in this sense, they share a common origin with the EAC-derived metabolites.



SCHEME 2

The lone example where the "heterolytic" pathway appears to be operational in an alga is in the brown seaweed *Cymathere triplicata* (45). When this Washington kelp is exposed at low tide, an odor of cucumbers can be detected. Indeed, during extraction of this alga, our co-workers in adjacent laboratories became convinced we were making cucumber salad! In this vein, *C. triplicata* has been examined for its potential as a source of edible kelp products (46,47). Examination of the lipid constituents led us to characterize 2*E*-nonenal and 2*E*,6*Z*-nonadienal from *C. triplicata* (48), the known odorant principles from cucumber (49). In this case, the product likely results from "heterolytic" hydroperoxide lyase reaction on 12-HpETE (**41**) to give a 9-carbon aldehyde and an 11-carbon oxo-acid (Fig. 3), or the equivalent set of reactions on a 10-hydroperoxy C_{18} fatty acid, and thus, reasonably involves the intermediacy of an EAC. As should be expected, the alga was also a source of several hydroxy acids, including 12*S*-HETE (**7**), 13*S*-HOTE (**20**) (see Scheme 2—compound structures 20–39), and 10*S*-HODTA (**21**), substantiating the presence of lipoxygenase metabolism in the alga. As detailed below, this same collection of *C. triplicata* yielded a fascinating array of bicyclic ethers and tetracyclic lactones (see below).

5. Lyngbyalactone from *L. majuscula*

In addition to the above reviewed C_{18} trioxilin-type metabolite (**13**) from the cyanobacterium *L. majuscula*, a Caribbean collection of this blue-green has yielded an epoxylactone of obviously related biogenesis (Todd, J.S., and Gerwick, W.H., unpublished observations). The structure of this new metabolite, lyngbyalactone (**22**), was assembled from a standard assortment of two-dimensional (2D)-NMR and mass spectral techniques. While partial relative stereochemistry could be deduced from coupling constants, absolute stereochemistry has not yet been assigned. The structure of lyngbyalactone is consistent with a biogenesis involving a ω6-LO followed by rearrangement of the hydroperoxide to an EAC and its capture through addition of the carboxylate to C_9 (Fig. 4).

6. Sarcolactone from *Sarcodiotheca gaudichaudii*

The coldwater red alga *S. gaudichaudii* collected from the state of Washington in the United States was recognized as a source of oxylipins from observation of characteristic blue charring compounds on TLC of the crude extract (48). Subsequently, we isolated two homologous hydroxy acids, 8*R*-HETE (**23**) and 8*R*-HEPE (**24**), as well as the homolytic-type hydroperoxide lyase product from 8-HpETE, trideca-2*E*,4*E*,7*Z*,10*Z*-tetraenal (see above).

Two additional products, related to one another as deriving from ω3- and ω6-fatty acids, were obtained and characterized as epoxylactones (**25**,**26**). Their planar structures were assembled from a combination of 2D-NMR and mass spectrometry (MS) data. Stereochemistry of the epoxide and olefins followed from coupling constant analysis while the *threo* relationship of the C_9 and C_{10} substituents was demonstrated by ^{13}C NMR analysis of the 9,10-acetonide which was formed by sequentially hydrogenating sarcolactone A (**25**) to the 8,9-epoxylactone, $LiAlH_4$ treatment to form the 1,9,10-trihydroxyalkane, and acetonide formation using 2,2-dimethoxypropane and *p*-toluenesulfonic acid. An absolute stereochemistry of 8*R*,9*R*,10*R* was established for **25** by selectively opening the lactone with dilute base, methylation to the methyl ester, formation of the *p*-bromobenzoate derivative followed by circular dichroism (CD) analysis (48).

From this array of metabolites, it appears that *S. gaudichaudii* possesses an operational 8-LO, a class of lipoxygenase only previously observed in starfish (15), corals (24), and a red alga related to *S. gaudichaudii* (66). Further, as is often the case, it appears that the resulting hydroperoxide may either be reduced, subject to homolytic cleavage with subse-

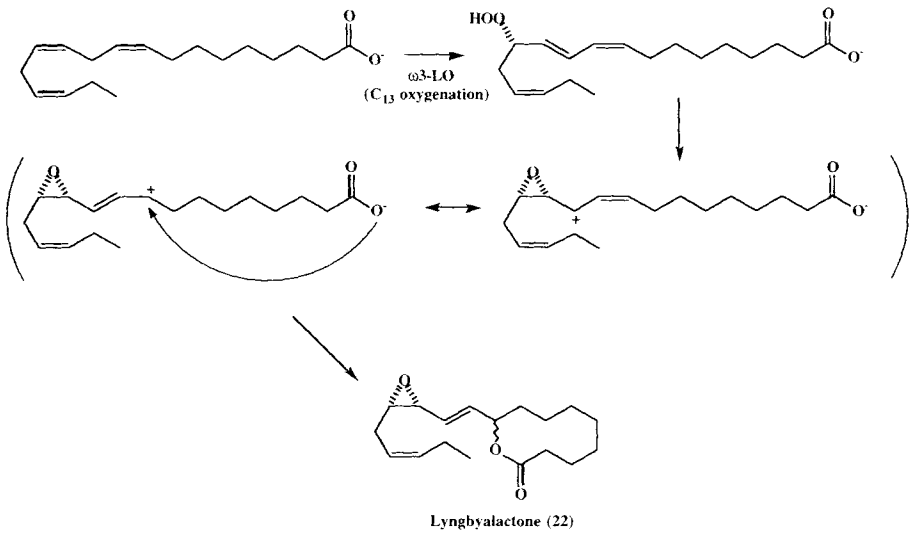


FIG. 4. Proposed biogenesis of lyngbyalactone (**22**) in the marine cyanobacterium *Lyngbya majuscula*.

quent carbon chain scission, or converted to an EAC which is trapped by the carboxylate. It is interesting to note the different fates of this proposed EAC in the two related red algae *S. gaudichaudii* and *Agardhiella subulata* (see Fig. 11 later in this paper).

7. Neodidemnilactone and Ascidiatrienolide from the Tunicate *Didemnum* spp.

The structures of three lactone-containing oxylipins from the tunicate *Didemnum moseleyi*, including neodidemnilactone (**28**), were reported and their structures confirmed by chemical synthesis (50). Neodidemnilactone is highly similar to the corrected structure of ascidiatrienolide (**27**) (51), differing only in the geometry of side-chain double bonds. Ascidiatrienolide was originally isolated from the related tunicate *D. candidum* (52). On the basis of extensive synthetic studies, the lactone ring size was modified in ascidiatrienolide from a nine-membered to ten-membered ring (51). The originally proposed structure of ascidiatrienolide was of the unusual ω7 structure class whereas the ten-membered lactone structure is more in line with established fatty acids (ω6), and predicts arachidonic acid as the ultimate precursor. Biogenetically, the presence of a 1,2 diol derivative adjacent to a conjugated triene in ascidiatrienolide (**27**) and neodidemnilactone (**28**) suggests an 8,9-epoxy-leukotriene A_4-type intermediate that is opened at C_9 by the carboxylate. This in turn could derive from an 8,9-epoxy allylic carbocation of the type proposed in this review, itself coming from 8-HpETE (Fig. 5).

8. Oxylipins from the Green Alga *Acrosiphonia coalita*

The Oregon green alga, *A. coalita*, has been a rich source of acyclic oxylipin metabolites (53). These are included in this review as they could be viewed as involving an EAC species, although, in this case a strong argument also can be made for an alkoxyl radical intermediate. An alkoxyl radical intermediate is suggested because this alga lacks lactone metabolites which logically derive from quenching of an intermediate carbocation with the carboxylate, as seen above and below for various oxylipins from other algae.

The metabolites of *A. coalita* include chain cleaved C_{10} compounds containing conjugated trienal and hydroxy functionalities (**29**) as well as the same functionality in C_{18} acids (**30**) (53). Intriguingly, this same relationship of functional groups was found in a chain-rearranged metabolite in which it is presumed that one carbon is excised from the C_{18} chain to form an aldehyde (**31**). This spectrum of metabolites is consistent with an initial metabolism of stearidonic acid by an ω10-LO (C_9 oxygenation). The hydroperoxide is then possibly subject to homolytic cleavage with ensuing carbon chain fragmentation to the α-methylene side to produce compounds such as **29**. Alternatively, rearrangement to an EAC with sequential migration of an α-epoxy proton, the carbon chain, and finally the epoxide could conceivably give the unsaturated aldehyde **31** (Fig. 6).

However, it is conceivable that all of these *A. coalita* metabolites could derive from a homolytic hydroperoxide cleavage with no ensuing formation of an EAC (17). For example, α-methylene cleavage of the alkoxyl radical could occur with subsequent carbonyl formation and a new C_8-C_{10} bond (Fig. 6). Abstraction of a hydrogen atom from what was formerly C_{10} would give a conjugated dienal or trienal, depending on the state of oxidation at the ω3 position, for at some point in the process, these metabolites become ω3-oxidized with concomitant introduction of the conjugated triene. As several of the *A. coalita* co-metabolites possessed *E,Z,E* conjugated trienes (12), which are consistent with the action

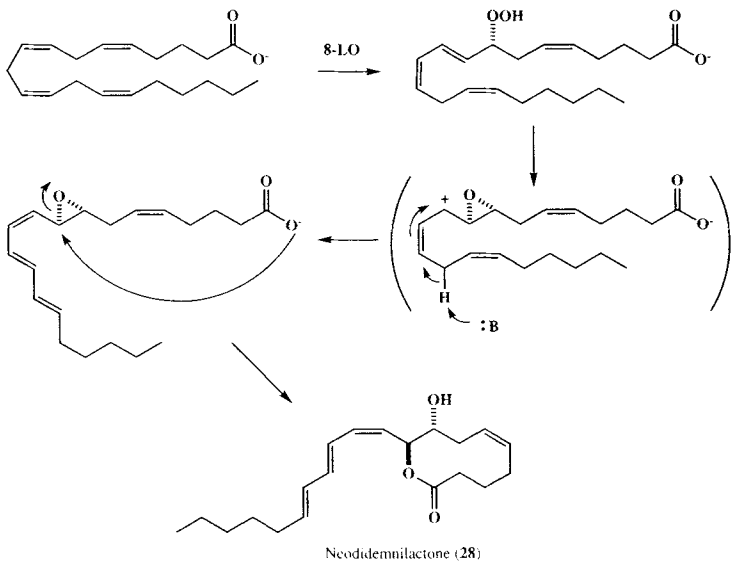


FIG. 5. Proposed biogenesis of neodidemnilactone (**28**) from the tunicate *Didemnum moseleyi*.

of an LO showing ω3 specificity, we propose this activity in the generation of metabolite **31** (Fig. 6). That radical chemistry is involved in hydroperoxide metabolism in *A. coalita* is further implicated by the isolation of four regio- and stereoisomeric epoxyalcohols which logically derive from the 9-hydroperoxide or 13-hydroperoxide. In each series, the locations of the epoxide and alcohol functionalities are consistent with rearrangement of an alkoxyl radical deriving from a homolytic O-O bond cleavage (17). However, as described above, a partially ionic mechanism to these epoxyalcohols cannot be ruled out in which an intermediate EAC is trapped with water.

CARBOCYCLIC OXYLIPINS—OXYLIPINS CONTAINING CYCLOPROPYL RINGS

1. Constanolactones from the Red Alga *Constantinea simplex*

The small mushroom-shaped Oregon alga, *C. simplex*, is a rich source of carbocyclic oxylipins of a cyclopropyl nature. The initial report of two such compounds, constanolactones A (**32**) and B (**33**) (54), has been expanded to a total of seven related products (31). The structural variation between these assorted metabolites involves ω3 and ω6 unsaturation, epimers at C_9 or C_{11}, and 1,4-diols (e.g., at C_9 and C_{12}, **32**) vs. 1,2-diols (e.g., at C_{11} and C_{12}, **34**). Planar structures and relative stereochemistry in these metabolites were determined chiefly by NMR while absolute stereochemistries were determined in most cases by either gas chromatography (GC)–MS analysis of malate fragments suitably derivatized as menthoxycarbonyl dimethyl esters or by CD analysis of benzoate esters (31).

Co-occurring with these carbocyclic oxylipins were the simple hydroxy-acids 12*S*-HETE (**7**) and 12*S*-HEPE (**8**)(54), thereby implicating a 12-LO-initiated biogenesis of the more complex carbocyclic metabolites. Their biogenesis previously has been speculated upon, and with the preponderance of lactone metabolites that this alga produces, likely involves an ionic mechanism in which an intermediate carbocation is trapped by the carboxylate. Furthermore, as all of the constanolactones are of 12*S* stereochemistry and we have shown that in constanolactone B only the 12-hydroxy group oxygen derives from molecular oxygen (55), there is good support for the 12-LO-initiated pathway. By this model (Fig. 7), hydroperoxide rearrangement to an EAC intermediate is succeeded by a potentially concerted electron shift to form the C_9-C_{10} olefin, C_6-C_8 cyclopropyl ring and δ-lactone. Speculation that this unsaturated epoxide may be the true end product of enzymatic metabolism which is then subject to nonenzymatic hydrolysis was supported by isolation of both epimers at C_9 in the 1,4-diols and both epimers at C_{11} in the 1,2 diols. Further, minor solvolysis products were isolated and characterized in which a C_9 methyl ether was presumably formed from this putative unsaturated epoxide metabolite during extraction with MeOH (31).

Accompanying these hydroxy acids and carbocyclic oxylipins was the chain cleaved "lyase" product 12-oxo-5*Z*,8*E*,10*E*-dodecatrienoic acid (**35**)(54). As this product likely results from cleavage between the hydroperoxide-bear-

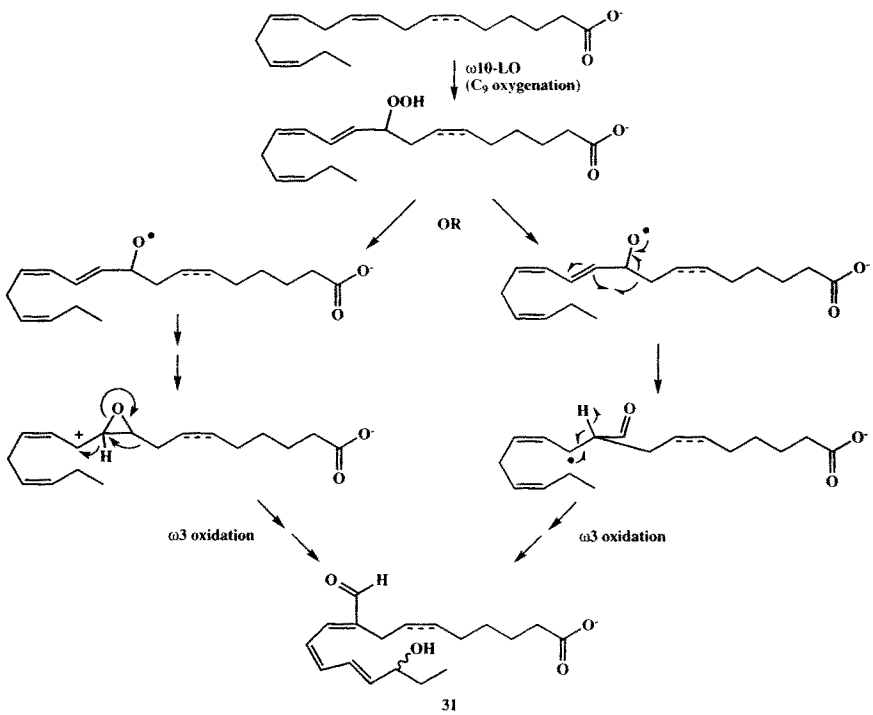


FIG. 6. Two proposals for the biogenesis of branched-chain trienal **31** from the green alga *Acrosiphonia coalita*.

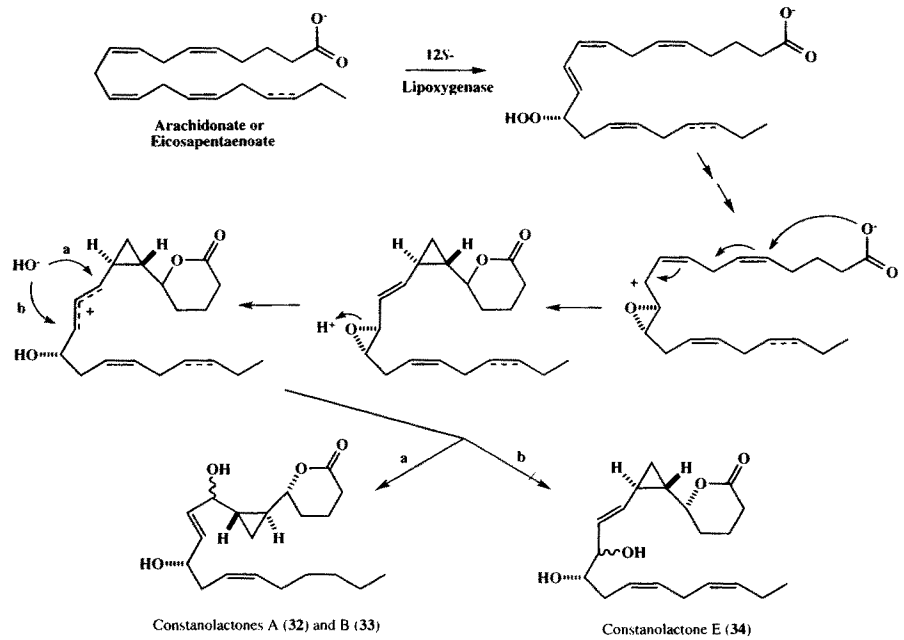


FIG. 7. Proposed biogenesis of 1,4-diol-containing constanolactones A and B (**32,33**), and 1,2-diol-containing constanolactone E (**34**).

ing carbon and α-methylene carbon (17), a radical process is implicated which would stem from a homolytic cleavage of the hydroperoxide, possibly as an early branch in the metabolism of hydroperoxides in *C. simplex* (e.g., see Fig. 3).

2. Neohalicholactones from a Sponge and a Brown Alga

Two closely related cyclopropyl ring-containing oxylipins, halicholactone (**36**) and neohalicholactone (**37**), were isolated from the Japanese sponge *Halichondria okadai* (56). Their planar structures were deduced by extensive NMR work and showed halicholactone to be a cyclopropyl lactone of the ω6 class while neohalicholatone was a comparable structure of the ω3 class. The *R* absolute stereochemistry at C_{15} was deduced for halicholactone (**36**) by comparison of the C_{14}-C_{20} fragment with an authentic standard. Subsequently, an X-ray study of neohalicholactone (**37**) provided relative stereochemistry in this series (57).

A potential complication in the structure of halicholactone and neohalicholactone was introduced by isolation and structural description of "neohalicholactone" from the Pacific brown alga *L. sinclairii* (58). From this kelp we had earlier isolated a variety of 15-lipoxygenase products, all of which had a 15*S* stereochemistry (e.g., 15*S*-HETE, **38**, see above) (9). Stereoanalysis of C_{15} in the *L. sinclairii*-derived "neohalicholactone" by conversion to the menthoxycarbonyl derivative, ozonolysis, methylation, and GC analysis showed this to be exclusively of *S* stereoconfiguration. Consideration of the proposals put forth for the biogenesis of neohalicholactone (5) led us to speculate that the C_{15}-stereochemistry would be unchanged by the biosynthetic process from the initially formed hydroperoxide.

As our isolate from *L. sinclairii* and that previously obtained from *H. okadai* had nearly identical NMR shifts and the same sign and similar magnitude of optical rotation, it was possible that the C_{15} stereochemistry as originally assigned was in error. However, clarification of this has come from synthetic studies on these metabolites (59), and indicates that the stereochemistry at C_{15} in sponge-derived neohalicholactone is correct as originally formulated (Wills, M., University of Bath, United Kingdom, personal communication). Correspondingly, it appears that the kelp-derived neohalicholactone is in fact diastereomeric to the sponge material at C_{15}. As such, it would be better referred to as "isoneohalicholactone" with its structure most accurately represented by compound **39**. Apparently, the C_{15} stereochemistry is remote enough from other stereocenters that it has minimal effect on the NMR features of the two molecules.

It is remarkable that the same functionalities and relationship of functionalities, as well as most of the relative stereochemistry, would be the same in metabolites coming from such disparate sources as red algae (**32**), brown algae (**39**), and a sponge (**36**). This finding suggests analogous biosynthetic processes at work with only the initial point of oxidation differing (*C. simplex*—12*S*-lipoxygenase; *H. okadai*—15*R*-lipoxygenase; *L. sinclairii*—15*S*-lipoxygenase).

CARBOCYCLIC OXYLIPINS—OXYLIPINS CONTAINING CYCLOPENTYL RINGS

1. Hybridalactone from the Red Alga *Laurencia hybrida*

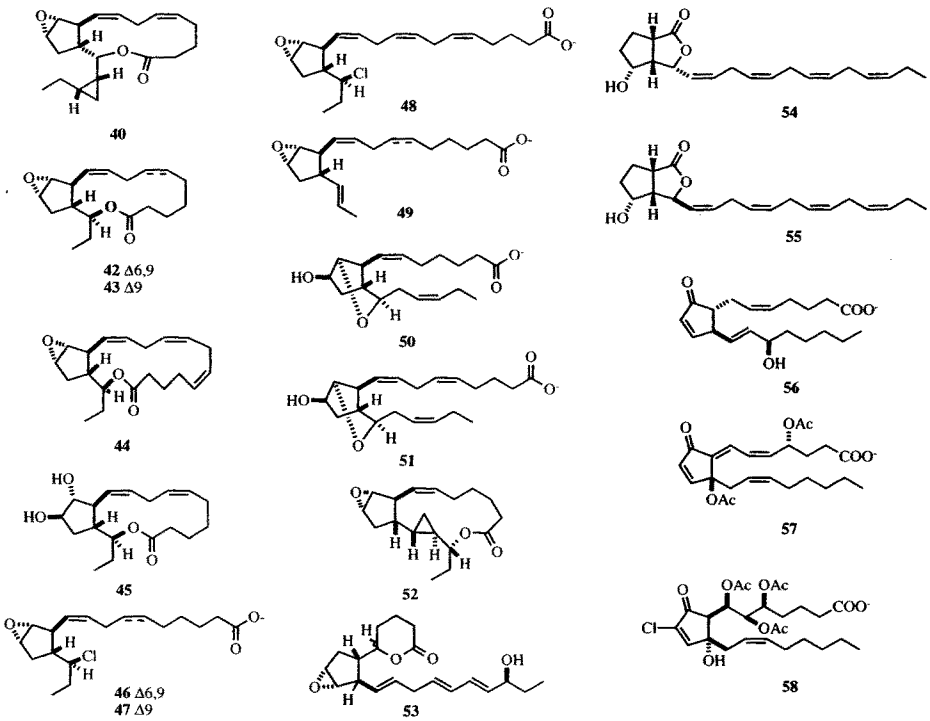
The red alga *Laurencia hybrida* was the first marine algal source of an unusual prostaglandin analog, hybridalactone

(**40**) (see Scheme 3, compound structures 40–50) (60), and its structure elucidation and other features of its chemistry have been previously reviewed (1,5). This unusual tetracyclic metabolite possesses not only a cyclopentane ring, but also cyclopropane, epoxide, and macrolactone rings. The position of the cyclopentyl ring is unique in that it bridges C_{10} and C_{14} rather than C_8 and C_{12} as in mammalian prostanoids. These various structural features led Corey and co-workers (30) to propose a 12-lipoxygenase initiated biogenetic route to hybridalactone (Fig. 8). In this case, rearrangement of the dienyl hydroperoxide (12*S*-HpETE, **41**) to an EAC is followed by carbocyclization to an allyl carbinyl cation. Following additional cationic rearrangements, a cyclopropyl carbinyl cation intermediate could be captured through nucleophilic attack at C_{15} by the carboxylate, yielding hybridalactone. This biogenetic proposal is strengthened as it correctly predicts the relative and absolute stereochemistry of hybridalactone beginning with 12*S*-HpETE (**41**). These stereochemical features of hybridalactone had been shown through total synthesis and an X-ray crystallographic study of a heavy atom derivative. Additional studies showed other 12-LO-derived metabolites [12*S*-HEPE (**8**)(36) and 12-oxo-5*Z*,8*E*,10*E*-dodecatrienoic acid (**35**)(61)] were present in this extract, providing further support for this 12-LO-initiated route to hybridalactone. Several of the ensuing biogenetic proposals for cyclopentane-containing oxylipins in this review are conceptually based on and similar to the template (Fig. 8) originally developed for hybridalactone (**40**). Finally, it should be noted that in this case, as in several others in this review, there is evidence of a possible branch in the hybridalactone metabolic pathway which departs at the point of alkoxyl radical formation (Fig. 3) and produces 12-oxo-5*Z*,8*E*,10*E*-dodecatrienoic acid (**35**).

2. Ecklonialactones from Two Brown Algae

The Japanese kelp *Ecklonia stolonifera* morphologically resembles the well known genus *Laminaria*, and as such, is a conceivably good food source for marine molluscs, including the abalone (*Haliotis* sp.). However, it appears that this alga is protected against rampant predation by herbivores through production of defensive natural products. These include, not surprisingly, phlorotannins, a natural product class which has demonstrated antifeedant roles in many macroalgae (62). A more unusual group of defensive compounds was found in the lipid-soluble materials from *E. stolonifera*, and includes a series of tricyclic (**42-44**) (63) and bicyclic ecklonialactones (**45**) (64). The latter bicyclic compound conceptually results from epoxide hydrolysis. These structures were assembled from detailed NMR analysis and confirmed by X-ray analysis of crystalline ecklonialactone A (**42**).

Interestingly, three of the same tricyclic metabolites previously reported from *E. stolonifera* were recently isolated by our group from the related Oregon "feather boa" kelp *Egregia menziesii* (both algae are classified in the order Laminariales) (65). The absolute stereochemistry of the series was established by selective opening of the epoxide at C_{13} in this latter isolate of ecklonialactone A (**42**), formation of the corresponding *bis*(*p*-bromobenzoate), and measurement of a negative exciton chirality in its CD spectrum.



SCHEME 3

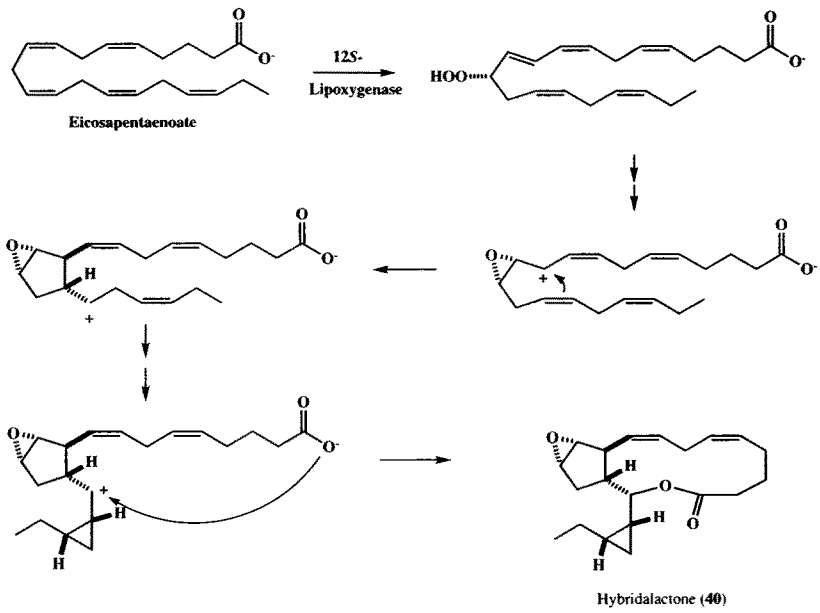


FIG. 8. Proposed biogenesis of hybridalactone (**40**) in the red alga *Laurencia hybrida* (adapted from Ref. 30).

The ecklonialactones are of obvious structural similarity to hybridalactone (see above, **40**) (30) which suggests that they may arise through an analogous series of biosynthetic events (Fig. 8). Furthermore, at comparable centers, they possess identical relative and absolute stereochemistries. The 18-carbon fatty acids stearidonic acid (18:4ω3) and α-linolenic acid (18:3ω3) are the likely precursors to ecklonialactones A (**42**) and B (**43**), respectively, while eicosapentaenoic acid (20:5ω3) is the apparent precursor to ecklonialactone E (**44**). LO oxidation of any of these precursor fatty acids at the ω6 position would yield a dienyl hydroperoxide which could rearrange to the proposed EAC (Fig. 9). In similarity to hybridalactone biogenesis, the carbocation is envisioned to induce carbocyclization, leaving a new carbocation at the ω3 position. In contrast to the remainder of the hybridalactone biogenetic pathway, this ω3 carbocation is directly captured by

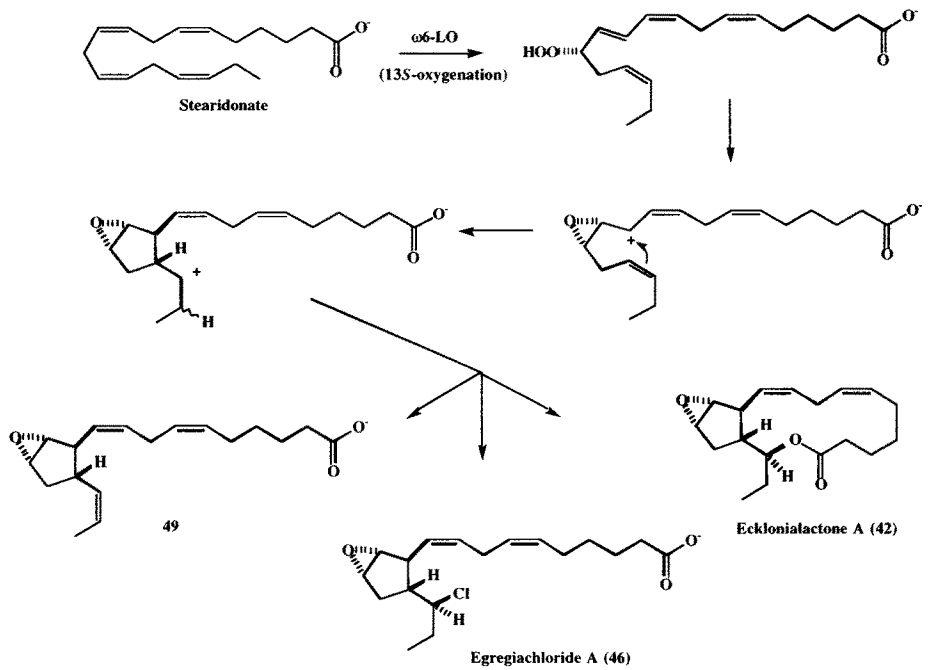


FIG. 9. Proposed biogenesis of ecklonialactone A (**42**), egregiachloride A (**46**), and egregia olefin derivative **49** in the brown algae *Ecklonia stolonifera* and *Egregia menziesii*.

the carboxylate and forms the macrolactone [14-membered ring for ecklonialactones A (**42**) and B (**43**), 16-membered ring for ecklonialactone E (**44**)]. As detailed subsequently, in *Egregia menziesii* there exist other routes for capture or quenching of this ω3 carbocation.

3. Egregiachlorides from *E. menziesii*

The Oregon brown kelp *E. menziesii* was a source of two series of carbocyclic oxylipins which conceivably derive *via* an intermediate EAC, the ecklonialactones (**42–44**, see above) (65) and the egregiachlorides (**46–48**) (32). The structures of the egregiachlorides were assembled from detailed NMR study in concert with the full data sets, and relative stereochemistry was indicated by nOe results and comparison of ^{13}C NMR spectral data with that of the ecklonialactones. Absolute stereochemistry in this series was not unequivocally established. However, on the basis of comparable rotations with the co-occurring ecklonialactones, they were proposed to be of the same enantiomeric series. A *trans*-ω2-olefin metabolite (**49**) of structure comparable to the egregiachlorides was also characterized.

Egregiachlorides A–C (**46–48**) logically derive from stearidonic acid, α-linolenic acid, and eicosapentaenoic acid, respectively. Clearly, they emanate from a similar biogenetic pathway as those producing the ecklonialactones as they possess cyclopentyl and epoxide rings at the same positions and with the same relative stereochemistries (Fig. 9). As both the C_{18} acids (**46,47**) and the C_{20} acid (**48**) are oxidized at the ω6 position, it suggests initiation of this pathway *via* a LO with ω6 specificity. The unique feature of these metabolites is the presence of a chloro functionality adjacent to the cyclopentyl ring. In the biogenesis of the ecklonialactones, this position develops positive charge which is nucleophilically captured by the carboxylate. Correspondingly, this same carbocation at the ω3 position could be captured by chloride ion to yield the egregiachlorides (Fig. 9). Alternatively, this ω3 cation could be quenched by a proton loss from the ω2 position to yield the olefin analog **49**.

4. Cymathere Ethers and Lactones from the Brown Alga *Cymathere triplicata*

As detailed above, *C. triplicata* collected from the Washington coast is rich in various hydroxy acids [12*S*-HETE (**7**), 13*S*-HOTE (**20**), and 10*S*-HODTA (**21**)] as well as odoriferous lyase products (2*E*-nonenal and 2*E*,6*Z*-nonadienal). Accompanying these simple products was a complex assortment of cyclized products, including two bicyclic ethers, cymathere ether A (**50**) and B (**51**) (45), and a new lactone, cymatherelactone A (**52**) (48). The planar structures of all three of these were assembled by careful 2D-NMR work in concert with a full spectrochemical data set. Relative stereochemistry was established by nOe measurements, coupling constants, and comparisons to model compounds. Absolute stereochemistries at the C_{10} alcohol in cymathere ether A (**50**) and the C_{16} alcohol in the lactone-opened derivative of cymatherelactone A (**52**) were established using the modified Mosher Ester method (48). Note, the absolute stereochemistry at C_{10} in this stereostructure for cymathere ether A is opposite that originally proposed (45), and leads us to present an alternative biogenetic pathway (Fig. 10) (48). Finally, absolute stere-

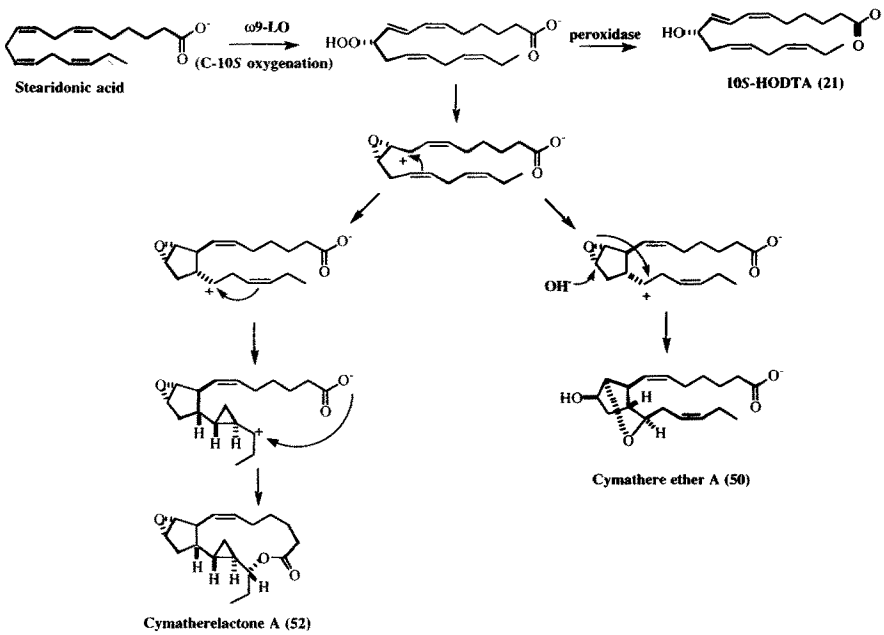


FIG. 10. Proposed biogenesis of cymathere ether A (**50**) and cymatherelactone A (**52**) in the brown alga *Cymathere triplicata*.

ochemistry at C_9 and C_{10} in the lactone metabolite **52** was separately determined by selective opening of the epoxide at C_{10} to the vicinal diol, derivatization to the corresponding *bis*(*p*-bromobenzoate), and measurement of a negative split Cotton effect (48).

The biogenetic origin of these various metabolites is likely quite similar to that proposed for the red algal metabolite hybridalactone (**40**, Fig. 8) (30). Beginning with the 18-carbon fatty acid stearidonic acid, we propose the initiating action of a ω9-lipoxygenase (C_{10} oxygenation) to 10*S*-HpODTA (Fig. 10). This is then either reduced by a peroxidase to 10*S*-HODTA (**21**) or rearranged to an epoxy allylic carbocation. This intermediate subsequently undergoes carbocyclization leaving a C_{13} carbocation. One fate for this cyclopentylcarbinyl cation is capture by the epoxide oxygen to form the C_9-C_{13} ether. This likely happens with concerted attack by water at C_{10}, yielding cymathere ether A (**50**). Another possible fate of the cyclopentylcarbinyl cation is migration of the ω3-double bond π-electrons to form the corresponding cyclopropylcarbinyl cation (Fig. 10). This apparently does not enter a "cyclopropyl-cyclobutyl-cyclopropyl manifold" as does hybridalactone (**40**) (30), but rather, is directly quenched by the carboxylate to form the tetracyclic macrolactone cymatherelactone A (**52**).

5. Agardhilactone from *Agardhiella subulata*

TLC analysis of extracts of the Atlantic red alga *A. subulata* showed the presence of blue-charring ultraviolet-active metabolites consistent with the occurrence of oxylipins. Isolation of one of these was hampered by its apparent instability as well as the co-occurrence of closely related and difficult to separate minor isomers. Nevertheless, repetitive high-performance liquid chromatography ultimately gave a greater than 95% pure sample which was amenable to structure elucidation by the combined techniques of 2D-NMR and MS (66). The new oxylipin, agardhilactone (**53**), is unique in several regards, including its highly functionalized skeleton (alcohol, diene, cyclopentyl ring, epoxide, and lactone ring). Moreover, the cyclopentyl ring is in a novel location relative to any other reported oxylipin, spanning C_6 and C_{10}. The presence and location of the epoxide, cyclopentyl, and lactone rings suggests a biogenesis for agardhilactone which hinges on the intermediacy of an EAC. In this case, initial LO oxidation is thought to occur at C_8, as in the related red alga *S. gaudichaudii*, followed by "hydroperoxide dehydrase"-type rearrangement to the 8,9-epoxide with C_{10} as an allylic carbocation (Fig. 11). Next, a flow of electrons from the 5,6-olefin to make the cyclopentyl ring and from the carboxylate to form the lactone is envisioned. Isomerization of the C_{11}-C_{12} olefin likely occurs at an early stage in the process, and then finally, a presumed 18-LO forms the diene and, following reduction, ω3 alcohol (12).

6. Bacillariolides from *Nitzschia pungens*

Marine diatoms have also been found as a source of unusual oxylipins, which in the case of *N. pungens*, may contribute to

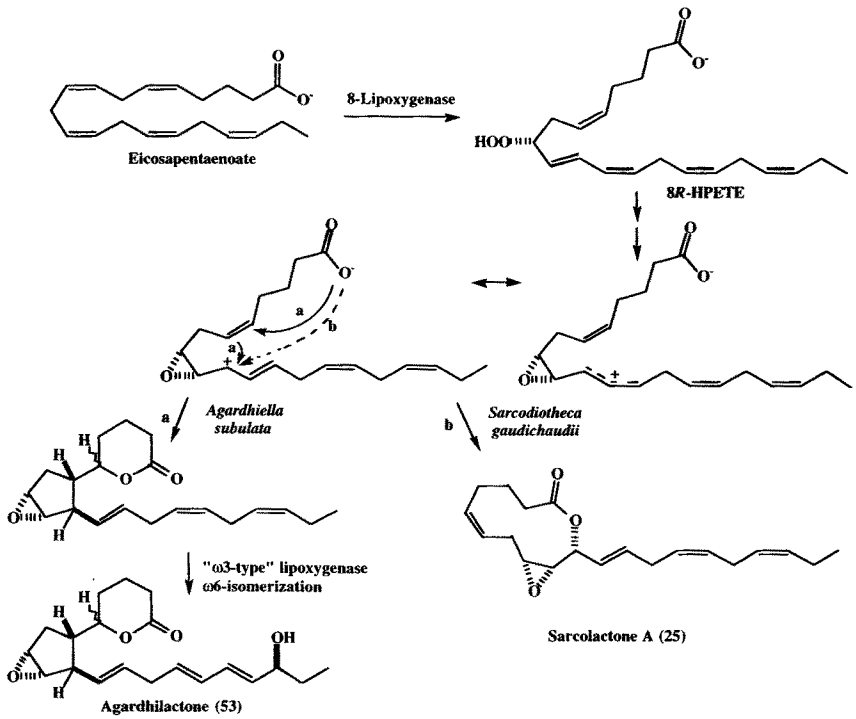


FIG. 11. Proposed biogeneses of agardhilactone (**53**) and sarcolactone A (**25**) in the related red algae *Agardhiella subulata* and *Sarcodiotheca gaudichaudii*.

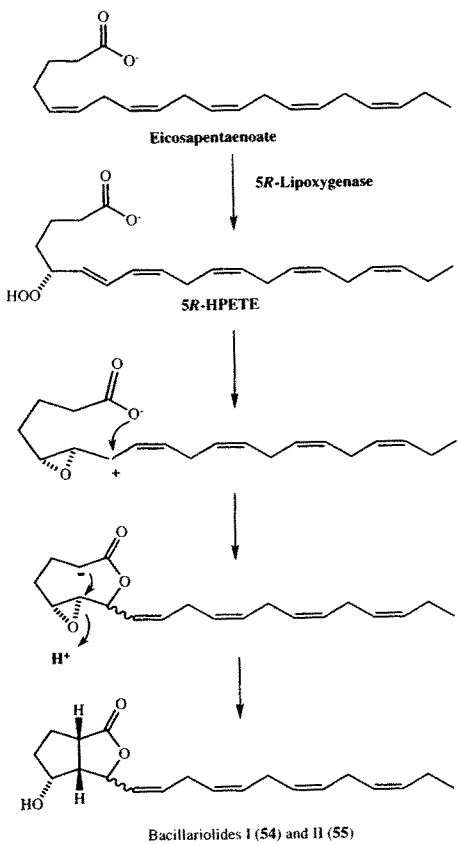


FIG. 12. Proposed biogenesis of bacillariolides I (**54**) and II (**55**) in the marine diatom *Nitzschia pungens*.

the severity of human intoxications resulting from shellfish consumed after blooms of this microalga (67,68). The structures of bacillariolides I (**54**) and II (**55**) were assembled from a normal interplay of NMR and other spectroscopic data while the absolute stereochemistry was established by X-ray crystallographic analysis of a chiral synthetic derivative (69). Biogenetically, it is conceivable that these metabolites are produced *via* a 5-lipoxygenase introduced hydroperoxide which is then converted to the corresponding epoxy allylic carbocation (Fig. 12). Following trapping of the cation by water to form the epoxyalcohol, or by the carboxylate to form an eight-membered lactone, the acidic proton at C_2 could be abstracted and the resulting anion could then attack the epoxide at C_6 to form the cyclopentyl ring and 5*S*-hydroxy group (Fig. 12). If an epoxyalcohol is an intermediate, condensation of the alcohol with the carboxylate at this point would yield the bacillariolide skeleton.

7. Clavulones, Puniglandins, and 15*R*- and 15*S*-Prostaglandins from Diverse Marine Corals

The final metabolites in this perspective are three series of related marine coral compounds: the 15*R*- and 15*S*-prostaglandins (**56**)(4,70), clavulones (**57**)(4,71), and puniglandins (**58**)(72). The 15-*epi*-prostaglandins such as 15*R*-prostaglandin A_2 (**56**), were the earliest group of prostaglandin-like materials to be discovered from marine organisms (73), and there has been tremendous subsequent interest in the mechanisms of their formation (23,24) and biological activity (4). Detailed reviews of these biosynthetic investigations have appeared previously (4,5).

While a definitive pathway from precursor polyunsaturated fatty acid to final prostaglandin A product has not been firmly established, elucidation of portions of the putative pathway has been described (Fig. 2) and involves conversion of an 8-lipoxygenase-derived polyunsaturated hydroperoxide into an allene oxide by the action of an allene oxide synthase (23,24)[hydroperoxide dehydrase (17)]. As described in the introduction to this perspective, formation of an allene oxide can be envisioned as involving an intermediate EAC which loses a proton, formally from C_9 in this case (Fig. 2). The allene oxide has a propensity to rearrange to a cyclopentenone product, which under enzymic influence, presumably occurs (but has not been shown) with stereocontrol. By either prior or subsequent oxidation at C_{15}, prostaglandins of the 15-*epi* series are produced from this LO-initiated pathway in the coral *Plexaura homomalla* (23,24). Puniglandins, such as puniglandin 1 (**58**) from the octocoral *Telesto reisii* (72), and clavulones such as clavulone I (**57**) from the soft coral *Clavularia viridis* (71), are structurally related prostanoid-like compounds with a proposed biogenesis quite similar to that suggested for the *P. homomalla* metabolites. As such, they too likely derive *via* pathways with intermediate formation of epoxy allylic carbocations (4).

CONCLUSIONS

The intermediacy of an epoxy allylic carbocation has been previously proposed in the biogenesis of a number of the above-reviewed marine oxylipins, both by ourselves as well as other workers in this field. However, as seen in this perspective on the biogenesis of a diversity of such metabolites, a more compelling case emerges for the central intermediacy and generality of an EAC. In some cases, the epoxide that is formed in this process is of *cis* configuration (e.g., **22,25, 40–44,46–49**) while in others a *trans* epoxide is produced (e.g., **3,9**). However, no discernable trend in the further metabolism or chemotaxonomy of these different epoxides is readily apparent. It is interesting to note the large number of cases in which metabolites hypothesized to emanate from a radical pathway and from a cationic pathway co-occur in the same alga. In this review, it is proposed that all of these products emanate from a single pathway, one which first involves formation of an alkoxyl radical and subsequently an epoxy allylic carbocation. From the first, a variety of chain-scission or oxygen rebound reactions occur which explain the frequent occurrence of short-chain aldehydes and epoxyalcohols in the algae. From the latter EAC intermediate, it is proposed that a variety of unusually oxidized and carbon chain-rearranged metabolites are produced.

Several interesting points arise through consideration of

the above general hypothesis. Chief among these is its highly testable nature. Evaluation of substrate analogs which carry heteroatoms or functionalities at key positions might be expected to inhibit this putative "hydroperoxide dehydrase." On the other hand, if there is as much mechanistic relatedness in these hydroperoxide rearrangements as herein speculated, this relatedness might also be mirrored in the biosynthetic enzymes and their corresponding gene sequences. Hopefully, we will see an increase in the exploration of these intriguing questions by biochemists, bioorganic chemists, and molecular biologists in the future.

ACKNOWLEDGMENTS

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Purification and Characterization of a 28 kDa Cytosolic Inhibitor of Cholesteryl Ester Hydrolases in Rat Testis

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ABSTRACT: A 28 kDa inhibitory protein was purified from rat testis cytosol by sequential 40–65% ammonium sulfate precipitation, cation exchange chromatography, anion exchange chromatography, and preparative SDS–polyacrylamide gel electrophoresis. The heat-stable, trypsin-labile protein exhibited nonenzymatic, concentration-dependent inhibition of testicular and pancreatic cholesteryl ester hydrolases at all stages of purification. Copurifying at each stage was a 26.5 kDa protein which comprised 25% of the mass of the two proteins. Polyclonal antibodies raised to either or both 28 kDa and 26.5 kDa proteins by direct injection of excised electrophoretic bands cross-reacted with both proteins on western blots, immunoprecipitated both proteins, and neutralized inhibitory activity. Amino acid compositions of the individual proteins electroeluted from SDS–polyacrylamide gels were different from those of other surface-active proteins of similar molecular weights. Both proteins exhibited identical pI of 4.8 on chromatofocusing columns and two-dimensional gel electrophoresis. Although the subcellular distribution of the 28 kDa protein is unknown, its testicular cytosolic concentration, calculated from the purified protein mass, was 8×10^{-9} mols/L, which probably underestimates the actual concentration by an order of magnitude. This is greater than the minimum concentration required for *in vitro* inhibition (10^{-9} mols/L), consistent with a physiological role for this protein.

Lipids 31, 1233–1243 (1996).

Catalytic activities of surface-active enzymes are known to be influenced by the presence of other proteins associated with the substrate surface. Well-characterized, physiologically relevant examples of such interactions include the activation of pancreatic lipase by colipase (1,2); activation of lipoprotein lipase by apolipoprotein C-II (3,4); activation of lecithin/cholesterol–acyltransferase by apolipoprotein A-I (5–7), and activation of bee venom phospholipase A_2 by mellitin (8). Other proteins are known to influence activity of hydrolytic enzymes by modifying the surface of lipophilic substrates (9–11). The pathophysiological significance of such proteins is manifest in the cytolytic potential of intracellular hydrolytic enzymes, which are generally characterized by broad substrate specificities, and in the general lack of understanding of mechanisms for regulating their activity *in vivo*. It has been suggested that accessory proteins may be involved in regulation of cholesteryl ester hydrolase (CEH) in various organs (12–18). However, there has been no report of the purification or specific characterization of such a putative regulatory protein.

Several investigators in this laboratory have noted unexpectedly high recoveries of catalytic activity or loss of activity at specific stages of purification of CEH isoenzymes from rat testis (19) or liver (20). These findings were suggestive of differential loss of inhibitory or stimulatory accessory factors during the course of purification. In this report, we describe the purification and characterization of a specific testicular cytosolic protein with properties consistent with a physiological role in regulation of CEH activity.

MATERIALS AND METHODS

Chemicals and supplies. Cholesterol [1-^{14}C]oleate (59.4 mCi/mmol) and Aquasol-2 were purchased from DuPont-New England Nuclear (Wilmington, DE); heptane [high-performance liquid chromatography (HPLC) grade], from Baxter (Columbia, MD); dialysis tubing, Gelman acrodiscs (0.22 μM) and other solvents, from Fisher Scientific (Norcross, GA); Immobilon-P Transfer Membranes (PVDF), from Millipore (Bedford, MA); SDS, acrylamide, *bis*-acrylamide, TEMED, ammonium persulfate, silver stain kit, electrophoretic molecular weight markers, gelatin, blotting grade GAR-HRP and blotting grade DAB, from BioRad (Melville, NY); BCA protein assay kit, from Pierce Chemical Co. (Rockford, IL); ultrapure $(NH_4)_2SO_4$ and Coomassie R-250, from ICN (Irvine, CA); porcine pancreatic CEH, trypsin-linked agarose beads and other chemicals and reagents, from Sigma (St. Louis, MO); electrophoretic equipment, from Hoeffer (San Francisco, CA); frozen rat testes (250–350 g Sprague-Dawley rats), from Zivic-Miller Laboratories (Zelienople, PA) and Hilltop Laboratories (Scottsdale, PA); FPLC Mono S (HR 5/5 and HR 10/10) cation exchange column, FPLC Mono Q (HR 5/5) anion exchange column, FPLC

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Abbreviations: ASP, ammonium sulfate precipitate; CEH, cholesteryl ester hydrolase; CIP-28, 28 kDa CEH inhibitory protein; HPLC, high-performance liquid chromatgraphy; I_{50}, amount of protein inhibiting 50% under assay conditions, evaluated from a protein concentration curve; PLAP, phospholipase-activating protein; PNP, *p*-nitrophenol; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.

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Superose 6 gel filtration column, FPLC Mono P chromatofocusing column (HR 5/20) and FPLC supplies, from Pharmacia (Piscataway, NJ); Bio-Gel TSK Phenyl-5PW HPLC column, from BioRad (Melville, NY).

Purification of CEH. Rat testicular cytosolic CEH was prepared according to Wee and Grogan (19). Testes were homogenized in buffer (1:2 wt/vol) containing: 20 mM Tris-HCl, pH 7.5; 250 mM sucrose; 80 mM KCl; 0.05% sodium cholate; 10 mM EDTA and 0.1 mM thioglycolate. Cytosol (S104) was prepared by differential centrifugation at 2000 × *g* (30 min), 10,000 × *g* (30 min), and 104,000 × *g* (90 min); brought to 40% saturation with $(NH_4)_2SO_4$; stirred for 30 min at 4°C and centrifuged at 10,000 × *g* for 30 min. Resulting ammonium sulfate precipitate (ASP) pellets were stored at −20°C until use. ASP was redissolved in 500 µL buffer (20 mM Tris-HCl, pH 7.5; 10 mM EDTA, 0.1 mM thioglycolate, 1 mM sodium cholate), desalted over Sephadex G-25 and used as a CEH source for inhibitor assays or further purified by cation exchange chromatography.

For further purification, the desalted sample (10–30 mg) was chromatographed on a Pharmacia Mono S HR 10/10 FPLC cation exchange column (4 mL/min), eluting with 40 mL of Buffer A (20 mM Tris-HCl, pH 7.5; 0.05% sodium cholate; 0.1 mM EDTA; 0.1 mM thioglycolate); 72 mL linear gradient, 0–14% Buffer B (Buffer A + 2 M NaCl); 24 mL 14% Buffer B; 1 mL linear gradient 14–100% Buffer B; 33 mL 100% Buffer B. Fractions (8 mL) were assayed for CEH activity. Fractions 13–15 were pooled and used as a CEH source for assays. EDTA (10 mM) was added to stabilize activity. Protein was estimated by the Pierce Bicinchoninic Acid method (21).

Purification of cytosolic CEH inhibitor. Supernatant from 0–40% ASP was adjusted to 65% saturation with $(NH_4)_2SO_4$ and stirred for 30 min at 4°C. ASP (40–65%) was collected by centrifugation at 10,000 × *g* for 30 min and stored at −20°C until needed. Each ASP pellet, corresponding to ten testes, was dissolved in 1 mL 20 mM Tris-HCl (pH 7.5, 0.1 mM thioglycolate, 10 mM EDTA), desalted on a preequilibrated Sephadex G-25 column, and used for inhibitor assays or further purified.

For further purification, desalted 40–65% ASP (50–70 mg protein) was centrifuged at 10,000 × *g* for 15 min at 4°C. Supernatant was chromatographed on a Pharmacia Mono S HR 5/5 FPLC cation exchange column, eluted at 1 mL/min with 5 mL buffer C (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA and 0.1 mM thioglycolate); 3 mL linear gradient, 0–16% buffer D (buffer C + 2 M NaCl); 10 mL 16% buffer D; 3 mL linear gradient, 16–40% buffer D; 7 mL 40% buffer D, 2 mL linear gradient 40–100% buffer D; 15 mL 100% buffer D. One mL fractions were desalted and assayed for inhibitory activity. Fractions 21–24 were pooled, desalted (Centriprep-10 or Centricon-10 concentrators; Amicon Corp., Danvers, MA) and used as a source of cation exchange purified inhibitor or for further purification. For anion exchange chromatography, cation exchange purified fractions 21–24 (50–300 µg protein) were chromatographed on a Pharmacia Mono Q HR 5/5 anion exchange column (1 mL/min), eluted with 5 mL buffer C; 3 mL linear gradient, 0–16% buffer D; 10 mL 16% buffer D; 3 mL linear gradient, 16–40% buffer D; 7 mL 40% buffer D; 2 mL linear gradient, 40–100% buffer D; 15 mL buffer D. One mL fractions were collected, desalted, and assayed for inhibitory activity.

Active fractions were concentrated by TCA precipitation or Centricon-10 microconcentrator, electrophoresed on 5–20% or 9–12% SDS–polyacrylamide gradient gels (22), and silver stained to assess purity, using BioRad low molecular weight standards for calibration.

CEH activity. Rat testicular neutral cytosolic CEH activity was assayed by the modified method of Wee and Grogan (19). Each assay contained 20 mM Tris-HCl, pH 7.5, 1 mM sodium cholate, 10 mM EDTA, 0.1 mM thioglycolate, and 10–25 µg 0–40% ASP protein or 5–10 µg cation exchange purified CEH in 500 µL total volume. Tubes were preincubated 5 min at the assay temperature (32 or 37°C). Cholesterol oleate (35 µM) labeled with 2–4 × 10^4 dpm cholesterol [1-^{14}C]oleate was added in 12.5 µL acetone, and incubation was continued for 20 min, unless otherwise stated. The reaction was terminated by adding 3.25 mL methanol/chloroform/heptane (1.4:1.25:1, by vol) and 50 µL of 1 N NaOH. Product oleate was measured in the aqueous phase by liquid scintillation counting as described by Ghosh and Grogan (23).

Porcine pancreatic CEH (Sigma) was assayed as described above by the modified method of Ghosh and Grogan (20), using 1 µg CEH and 5 mM sodium taurocholate (24). Activity of all enzymes was linear with protein concentration and time of incubation.

Measurement of inhibitory activity. CEH activity was measured with and without 40–65% ASP, cation exchange purified fractions 21–24, anion exchange purified fractions 21–24, or other inhibitor sources. Inhibitor was added just prior to addition of substrate. Inhibitory activity is expressed as %inhibition = 100 × (activity without inhibitor − activity with inhibitor)/activity without inhibitor; or units (1 unit = I_{50}, amount of protein inhibiting 50% under assay conditions, evaluated from a protein concentration curve).

Electroelution. Proteins in inhibitory fractions (1.7–2 mg cation exchange purified protein or 100–150 µg anion exchange purified protein) were electrophoresed on a 9–12% SDS–polyacrylamide gradient gel. Bands corresponding to Coomassie stained 25–31 kDa markers were cut from the gel and protein electroeluted into 0.04 M Tris-Acetate, 0.002 M EDTA, pH 8.6, using a Shandon (Cheshire, England) electroeluter for 3 h at 50 mA. Electrode buffer was 0.4 M Tris-Acetate. Excess SDS was removed by extensive dialysis against 0.04 M Tris-HCl, pH 7.5. Prior to assay, sample was adjusted to pH 7.5 with dilute acetic acid. For individual bands, 28 or 26.5 kDa proteins were prestained with $CuCl_2$ (25) and destained before electroelution (26).

Phosphatase assay. Hydrolysis of *p*-nitrophenylphosphate (20 mg/mL) was measured spectrophotometrically at 410 nm (27) using assay conditions described for testicular and pancreatic CEH. The reaction was terminated by addition

of 600 µL of 0.5 M phosphate buffer, pH 7.5 and 10 mM EDTA.

Hydrophobic chromatography. Pooled cation exchange fractions 21–24 (800 µg protein) were concentrated by 65% $(NH_4)_2SO_4$ precipitation and resuspended in 1 mL 0.1 M sodium phosphate, pH 7.0, 2 M $(NH_4)_2SO_4$. The 100 µL was chromatographed on a Biogel TSK-phenyl 5PW HPLC column pre-equilibrated with the same buffer and eluted (1 mL/min) by a 60 min decreasing linear $(NH_4)_2SO_4$ gradient (2 to 0 M); followed by 20 min elution with distilled H_2O.

Production of polyclonal antibody. For primary immunization, 650 µg cation exchange purified protein (pooled and concentrated fractions 21–28) was electrophoresed on a 9–12% SDS–polyacrylamide gradient gel. The gel was stained briefly with Coomassie R-250, to visualize bands corresponding to 26.5, 27, and 28 kDa and washed to remove excess stain. Selected bands were excised, homogenized in phosphate buffered saline and Freund's complete adjuvant (1:2:2, gel–saline–adjuvant). An emulsion was generated by repeated passage between two ground-glass barreled syringes and injected intradermally at 10–15 sites on the back of a New Zealand rabbit on day 0. On days 25 and 72, booster immunizations of 300–500 µg anion exchange purified protein (fractions 21–28) were injected with Freund's incomplete adjuvant. Ten days after the third injection, serum was tested for antibodies by Western blot analysis as described below. Additional boosters were given in incomplete adjuvant 7–12 d prior to subsequent bleedings. Individual rabbits were injected with all three bands, the 28 kDa band alone, or the 26.5 kDa band alone. Preimmune serum was collected 7 d before primary immunization. IgG was prepared from immune and preimmune serums by PEG precipitation (28).

Western blot analysis. Proteins separated by SDS–PAGE (9–12% gradient) were transferred to Millipore Immobilon-PVDF membrane by semi-dry blot transfer (Integrated Separation System, Natick, MA) for 1 h at 2 mA/cm^2, according to the manufacturer's instructions. Western blot analysis was carried out as described previously (29) using 1:300 dilution of IgG (1.3 mg/mL).

Immunoprecipitation. Inhibitory proteins were immunoprecipitated from cation exchange purified inhibitor (fractions 21–28) by the modified procedure of Kessler (30,31) using antibody complexed with purified protein-A conjugated polyacrylic beads (Sigma). Beads were precoated with gelatin to decrease nonspecific binding to beads, washed to remove excess gelatin, incubated for 1 h with immune IgG or preimmune IgG, centrifuged to remove excess IgG and incubated for 1 h with inhibitor-containing fractions or buffer. Beads were then centrifuged and the supernatants collected and assayed for inhibitory activity against pancreatic CEH.

Amino acid analysis. Cation exchange purified protein (25 µg) was electrophoresed on a 9–12% SDS–polyacrylamide gradient gel, electroblotted to Millipore PVDF membrane (32), and stained with Coomassie blue R-250. Stained proteins corresponding to the two major bands (28 and 26.5 kDa) and the minor 27 kDa were excised. Quantitative amino acid analysis was carried out by Hewlett-Packard Aminoquant Methodology in the Protein Core Facility at Virginia Commonwealth University. The amino acid compositions for mature rat serum apoA-I and PLAP were translated from the reported cDNA sequences (33,34). The amino acid composition of the 28 kDa estrogen-regulated protein of Leydig cells is published (35,36,37).

Two-dimensional gel electrophoresis. Using the method of O'Farrell (38), the cation exchange purified inhibitor 200–300 µg fractions 21–24) was separated by nonequilibrium pH gradient gel electrophoresis (5.5 h at 550v) in the first dimension and 10% SDS–PAGE in the second dimension. Gels were then silver stained or transferred to PVDF membrane for Western blot analysis. Protein molecular weight and pI were compared to the migration of two-dimensional electrophoresis markers (Sigma) (pI range 7.6–3.8; MW range 17,000–89,000).

Chromatofocusing. The 200–300 µg cation exchange fractions 21–24 were chromatographed on a Pharmacia FPLC Mono P HR 5/20 column eluted (1 mL/min) with a pH 6–4 linear gradient, consisting of 3 mL 0.025 M *bis*-Tris-HCl, pH 6.3, 0.1 mM EDTA, 0.1 mM thioglycolate, followed by 39 mL 1:10 Pharmacia Polybuffer 74-HCl, pH 4.0, 0.1 mM EDTA, 0.1 mM thioglycolate. Inhibitory activity was assayed in 1 mL fractions.

Gel permeation chromatography. Cation exchange purified protein (200 µg) was chromatographed on an FPLC Superose 6 gel permeation column eluted (0.2 mL/min) with 20 mM Tris-HCl, pH 7.5, 0.1 mM thioglycolate, 10 mM EDTA, 1 mM sodium cholate, and 150 mM NaCl. Inhibitory activity was assayed in 0.5 mL fractions.

RESULTS

Testis contains a temperature-labile CEH, which is active at 32°C and inactive at 37°C, and a temperature stable enzyme, which is active at both temperatures (39,40). Both activities precipitate from rat testis cytosol in 40% ammonium sulfate (41). As seen in Figure 1 and Table 1, the 40–65% ASP inhibits both forms of testicular CEH. Although the I_{50} for inhibition of the temperature-stable component (72 µg in Fig. 1A), which persists at 37°C, is lower than that for inhibition at 32°C (580 µg in Fig. 1A), this difference may not reflect differential sensitivities of the enzymes to inhibition (see the Discussion section). The inhibition is concentration-dependent, saturable (Fig. 1A), and reversible by dilution (data not shown), suggesting a reversible binding phenomenon. The principal inhibitory activity is trypsin-labile (Fig. 2B) and retained by dialysis membrane (Fig. 2C), indicative of a protein with molecular weight >12,000. However, the inhibition is heat-stable (Fig. 2A), independent of incubation time (Fig. 3), and independent of preincubation time with CEH (data not shown), indicating a nonenzymatic mechanism. Moreover, porcine pancreatic CEH was also inhibited by this factor (Fig. 1B), suggesting a general mechanism for inhibition. Pancreatic CEH provided a more homogeneous and stable enzyme

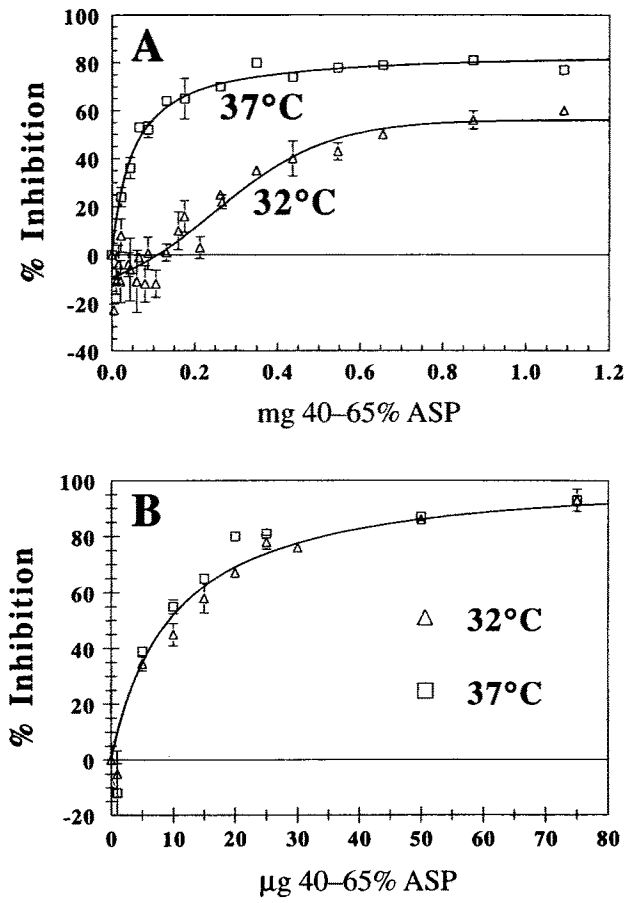


FIG. 1. Concentration-dependent inhibition of rat testicular cholesteryl ester hydrolase (CEH) (A) and pancreatic CEH (B) at 32°C (△) and 37°C (□) by the 40–65% ammonium sulfate precipitate from rat testis cytosol. Values are mean percentage inhibition of CEH control ± SEM (n = 3). Error bars not seen are contained within symbols.

model for mechanistic studies than the testicular CEH. The I_{50} for pancreatic CEH (10 µg in Fig. 1B) was somewhat lower than that for the temperature-stable testicular CEH and did not differ at 32 and 37°C, indicating that the inhibition is not intrinsically temperature-dependent.

The 40–65% ASP was further fractionated by cation exchange chromatography, yielding two major peaks of inhibitory activity in fractions 9–12 and fractions 21–28, respectively (Fig. 4A). Fractions 21–28 not only contained most of the inhibitory activity but were also much higher in specific inhibitory activity than any other fractions (Fig. 4B). As with 40–65% ASP (cf. Fig. 1A), cation exchange purified inhibitor was somewhat less effective in inhibiting testicular CEH at 32°C than at 37°C (Table 1). Although inhibition showed similar concentration dependency to that seen with 40–65% ASP (Fig. 5A), specific inhibitory activity was substantially increased (I_{50} = 10 µg in Fig. 5A; cf. 5.4 µg for 1 µg pancreatic CEH, not shown). Negative inhibition at very low inhibitor concentrations reflects activation, which persisted at every stage of purification, although it was not always apparent in the range of concentrations depicted.

Inasmuch as the testicular CEH is inhibited by phosphatase (41,42), general phosphatase activity was measured in cation exchange fractions (Fig. 4C). Coelution of the first peak of inhibitory activity with a major peak of phosphatase activity suggested that the inhibitory activity of interest was contained in the second, larger peak, which had no appreciable phosphatase activity. Thus, fractions 21–28 with 41–56% of the ASP inhibitory activity (Table 1) were used in subsequent purification steps.

Cation exchange fractions 21–28 were pooled and further fractionated by anion exchange chromatography. As seen in Figure 6, most of the inhibitory activity eluted in fractions 21–24. Although activity was assayed with the pancreatic CEH in the typical elution profile shown in Figure 6, a similar pattern was seen with testis CEH (data not shown). Moreover, specific inhibitory activities did not differ significantly between pancreatic (typically, 900 units/mg) and testicular CEH (Table 1) or between temperature-stable and temperature-labile CEH (Table 1) at this stage of purification. Inhibition by pooled anion exchange fractions (Fig. 5B) was also

TABLE 1
Purification of 28 kDa CEH Inhibitory Protein (CIP-28) from Rat Testis Cytosol (S-104)[a]

	32°C				37°C			
Purification step	Total recovery (units)	Specific activity (units/mg)	Fractional recovery (%)	Fold	Total recovery (units)	Specific activity (units/mg)	Fractional recovery (%)	Fold
40–65% $(NH_4)_2SO_4$ precipitate (ASP)	56	0.75	100	1	124	16	100	1
Cation exchange FPLC (fractions 21–28)	23	56	41	75	70	215	56	13
Anion exchange FPLC (fractions 21–28)	9	847	18	1129	9	821	8	51
SDS-PAGE of pooled cation exchange fractions (electroelution)	6	1898	11	2531	7	1866	5	117

[a]Inhibitory activity was measured with partially purified testicular cholesteryl ester hydrolase (CEH), as described in the Materials and Methods section. Inhibition of pancreatic CEH was similar to that of testicular CEH at each stage of purification. Total recovery is referenced to 10 testes; SDS–PAGE, SDS-polyacrylamide gel electrophoresis.

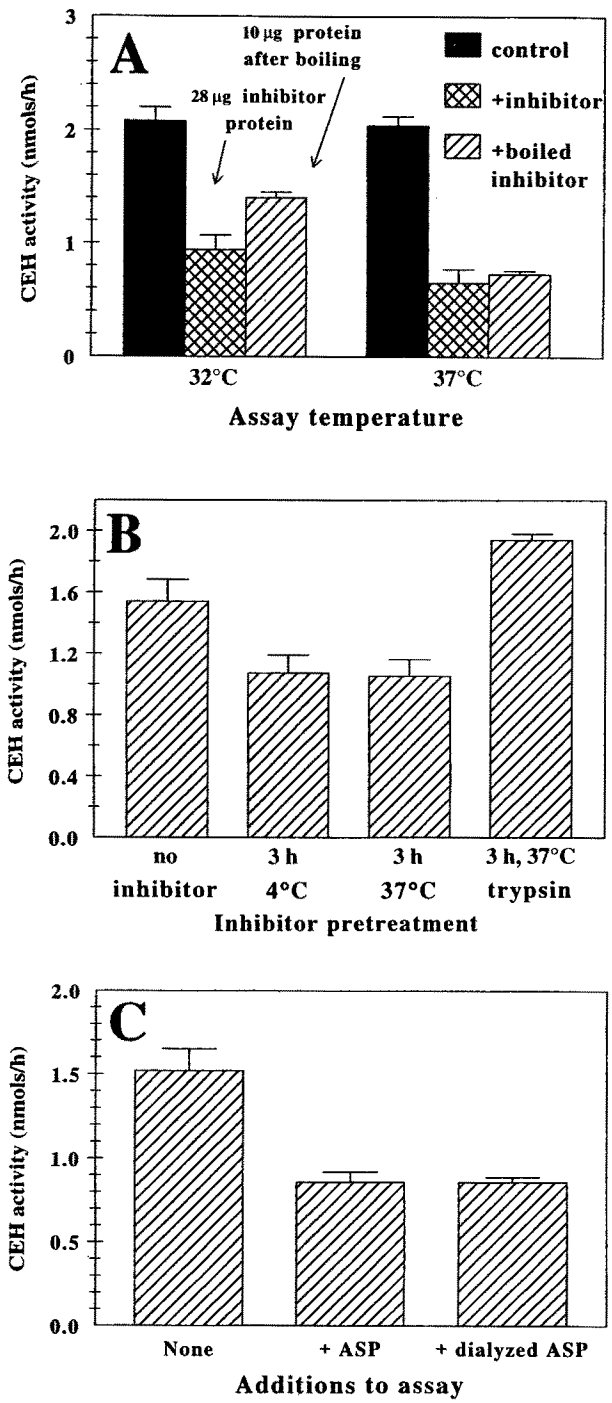


FIG. 2. Characterization of the inhibitory factor as a heat-stable protein. (A) Cation exchange purified inhibitor was boiled for 10 min and precipitated protein removed by centrifugation at 10,000 × *g* for 30 min (see Fig. 10 for recovery of protein after boiling). Rat testicular CEH was assayed at 32 or 37°C with buffer (control), with inhibitor or with equivalent supernatant from boiled inhibitor. (B) Cation exchange purified inhibitor was incubated for 3 h at 4°C or at 37°C in presence or absence of trypsin-linked agarose beads. Samples were then centrifuged at 12,000 × *g* for 5 min. Pancreatic CEH was assayed with or without 300 µL of each supernatant. (C) 40–65% ammonium sulfate precipitate (ASP) was dialyzed (M_r cutoff = 12 kDa) against 6 × 2 L assay buffer. Rat testicular CEH was assayed with or without 520 µg ASP, dialyzed or undialyzed. Values are mean percentage inhibition of CEH control ± SEM (n = 3). See Figure 1 for other abbreviation.

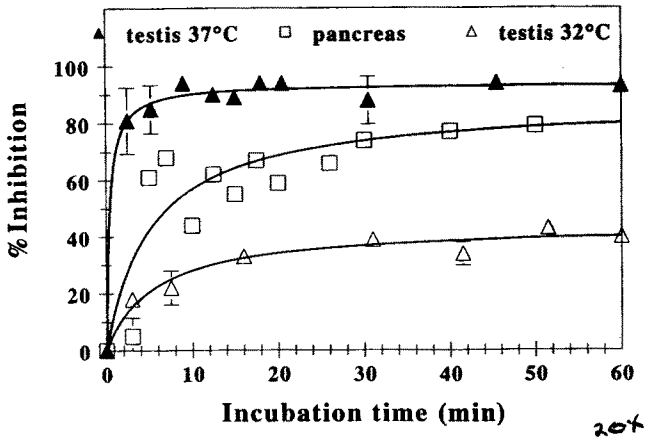


FIG. 3. Time-dependent inhibition of rat testicular CEH or pancreatic CEH by testicular cytosolic CEH inhibitor. Cation exchange purified testicular CEH or pancreatic CEH were assayed with and without cation exchange purified inhibitor (fractions 21–24) for the times indicated. Values are mean percentage inhibition of CEH control ± SEM (n = 3). Error bars not seen are contained within symbols. See Figures 1 and 2 for abbreviations.

concentration-dependent and produced a saturation curve similar to that of the 40–65% ASP and cation exchange purified fractions, whereas specific inhibitory activity was further increased (I_{50} = 3.3 µg in Fig. 5B). As in earlier stages of purification, very low inhibitor concentrations activated CEH.

Homogeneity was assessed by SDS–PAGE at each stage of purification (e.g., Figs. 7–8). However, none of the procedures resulted in a completely homogeneous protein. Moreover, purification steps beyond cation exchange gave poor recovery of total activity (see Table 1). Efforts to further purify the inhibitor by gel filtration chromatography produced a diffuse pattern of elution, indicating aggregation, even in the presence of bile salts, with no increase in specific activity (not shown). Hydrophobic interaction chromatography of ion-exchange fractions indicated that the inhibitor was hydrophobic but produced no appreciable increase in homogeneity (not shown).

Although none of the above purification procedures yielded a completely homogeneous protein, silver staining of SDS–polyacrylamide gels revealed a strong band at M_r 28 kDa in active fractions at each stage of purification (Figs. 7A and 8A). The electroeluted proteins from this band exhibited concentration-dependent inhibition of testis CEH (Fig. 5C), indicating presence of the inhibitory factor. Although the inhibition appeared to saturate at lower concentrations with the electroeluted inhibitor (by extrapolation: 40%, at 37°C; 23%, at 32°C) than with less homogenous preparations (typically 80–100%), I_{20} values were consistent with a high degree of enrichment (74 ng, 2, 8, and 20 µg, for electroelution, anion exchange, cation exchange, and ASP-purified inhibitor, respectively, assayed at 37°C). Moreover, very low concentrations of the less highly purified inhibitor also saturated at lower levels of activity (data not shown).

On narrow gradient (9–12%) SDS–PAGE, the broad 28

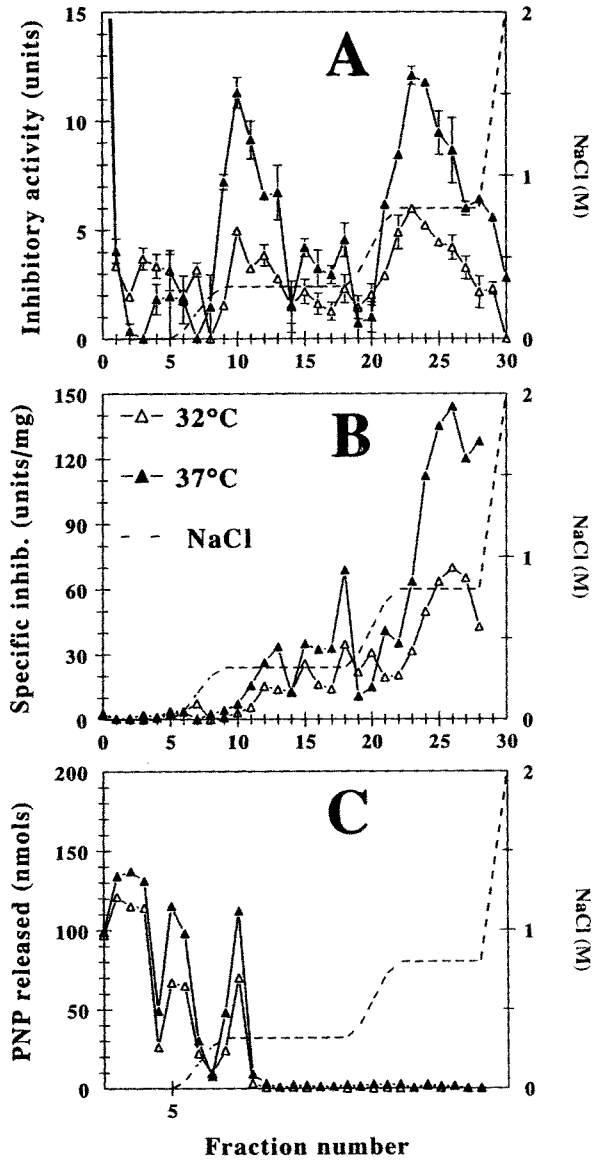


FIG. 4. Elution of CEH inhibitory and phosphatase activities from a cation exchange (FPLC Mono S) column (Pharmacia, Piscataway, NJ). Of each fraction 200 µL was assayed for inhibitory activity toward 22 µg cation exchange purified rat testicular CEH (A and B) and for pNPP hydrolytic activity (C) at 32 and 37°C. Panel A depicts units CEH inhibitory activity per fraction (purified from ten testes); panel B, specific inhibition per fraction; panel C, nmols *p*-nitrophenol (PNP) released per fraction. Values are means ± range (duplicates). Experiment was repeated with similar results. See Figure 1 for abbreviation.

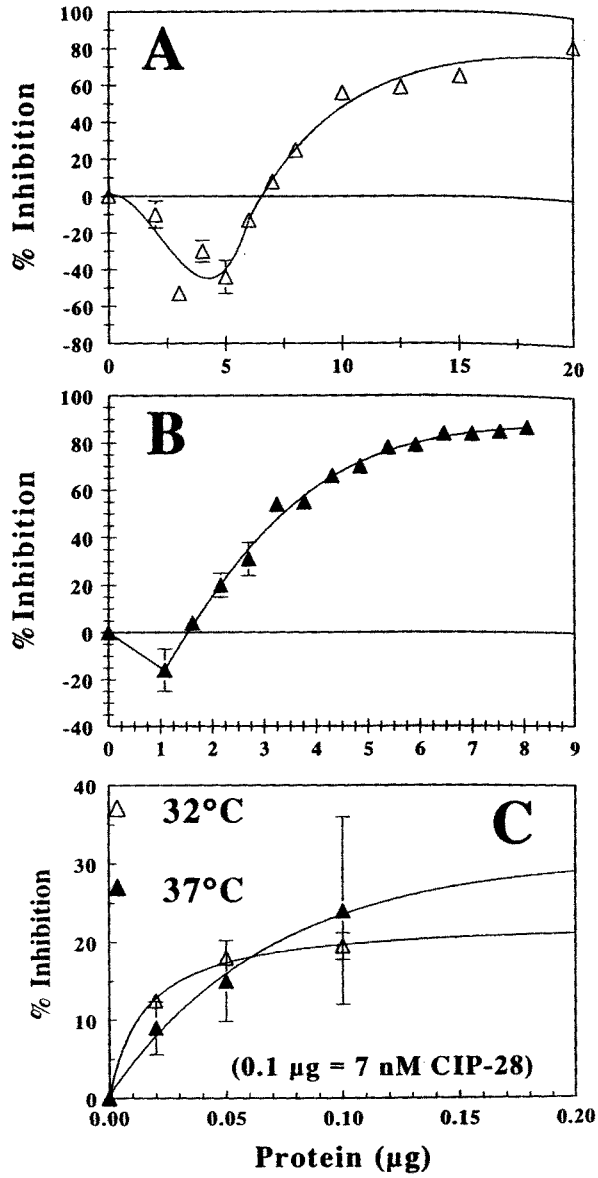


FIG. 5. Concentration-dependent inhibition of CEH by testis cytosolic inhibitor at various stages of purification. (A) Rat testicular CEH (3 µg desalted 0–40% ASP) was assayed at 32°C with cation exchange purified inhibitor (fractions 21–24); similar results were obtained at 37°C. (B) Pancreatic CEH was assayed with anion exchange purified protein (fractions 21–23); similar results were obtained with testicular CEH. (C)14.5 µg cation exchange purified rat testicular CEH was assayed at 32 and 37°C with varying amounts of electroeluted 28, 27, and 26.5 kDa proteins. Values are mean percentage inhibition of CEH control ± SEM (n = 3). Error bars not seen are contained within symbols; CIP-28, 28 kDa cytosolic inhibitor. See Figure 1 for other abbreviation.

kDa band was resolved into 28 and 26.5 kDa bands and a band at 27 kDa, which was not always visible (Figs. 8B and 9). The individual proteins could not be electroeluted in sufficient quantities to assay inhibitory activity. However, amino acid mass and composition (Table 2) were obtained for each of the three electroblotted, excised proteins of interest. The 28, 26.5, and 27 kDa proteins were present in the approximate molar ratio, 12:4:3. Consistent with their hydrophobic character, these proteins were relatively high in hydrophobic amino acids. The amino acid compositions were compared with those reported for other surface-active proteins of similar molecular weight, including Apolipoprotein A-I, phospholipase-activating protein (PLAP), and estrogen-regulated protein from Leydig cells (Table 2). Although none of the amino acid compositions were identical, Asx, Tyr, Val, Leu, Ile, Phe, Met, Pro, and Thr were equivalent in the 28 and 26.5 kDa pro-

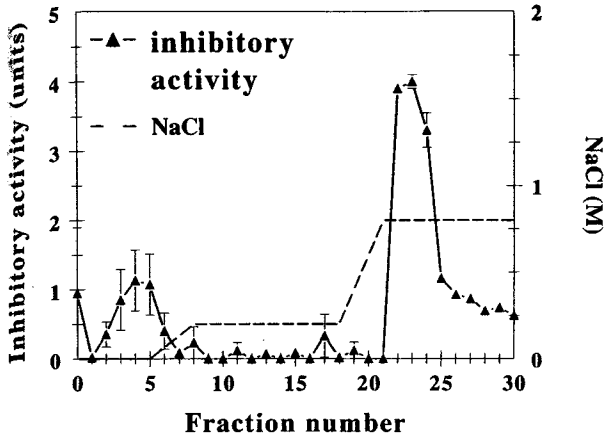


FIG. 6. Elution of cation exchange purified CEH inhibitor from an anion exchange column. Fractions 21–24 of cation exchange purified inhibitor were refractionated by anion exchange (FPLC Mono Q; Pharmacia). Of each fraction 400 μL was desalted and assayed for inhibitory activity against pancreatic CEH. Activity is reported as total units inhibitory activity per fraction (purified from 20 testes); values are mean percentage inhibition of CEH control ± SEM (n = 3). Error bars not seen are contained within symbols. See Figure 1 for other abbreviation.

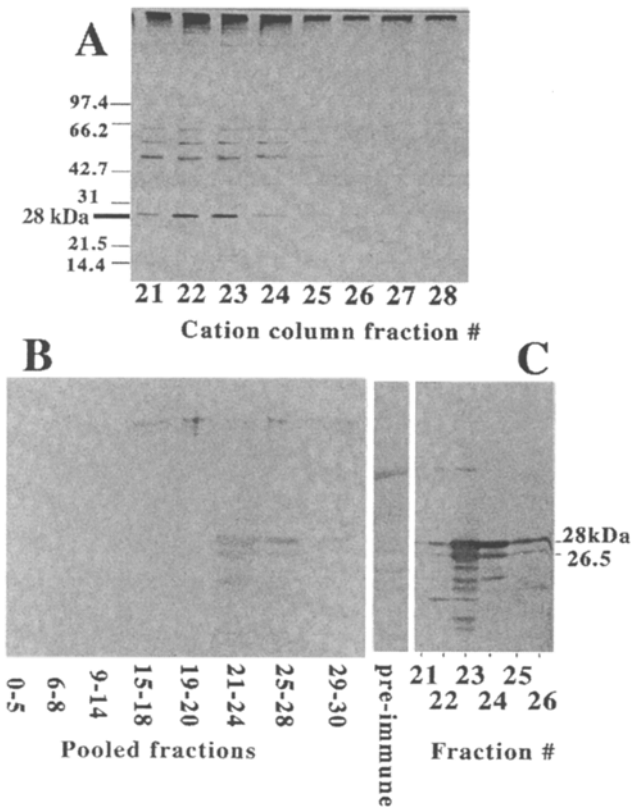


FIG. 7. Analysis of cation exchange fractions by SDS–polyacrylamide gel electrophoresis. Panel A: Silver stained 5–20% SDS–polyacrylamide gradient gel showing prominent 28 kDa band in fractions 21–28, corresponding to elution of inhibitory activity from cation exchange column. Positions of BioRad molecular weight markers are provided for comparison. Panel B: Western immunoblot of pooled cation exchange fractions, electrophoresed on 9–12% SDS–polyacrylamide gradient gel, transblotted to PVDF membrane and probed with antibody to 28 and 26.5 kDa proteins. Immunoreactive proteins at 26.5 and 28 kDa are resolved by the shallow gradient and coelute with inhibitory activity (see Fig. 4). Panel C: Western immunoblot of individual cation exchange fractions showing elution of immunoreactive 26.5 and 28 kDa proteins in fractions containing inhibitory activity (see Fig. 4). Other cross-reacting bands were also immunoreactive with preimmune sera, which did not react with 28 and 26.5 kDa proteins.

teins. Attempts to obtain primary sequences for these proteins were unsuccessful. Both proteins appeared to be *N*-terminally blocked.

Rabbit antibodies were raised by direct injection of the bands from SDS–PAGE, in combination or separately. As seen in Figure 10, strongest antibody titer was achieved with the combined proteins (lane 1), and antibodies raised to the individual 26.5 kDa (lane 2) or 28 kDa (lane 3) proteins exhibited mutual cross-immunoreactivity, further evidence that these proteins are closely related. In contrast, immunoreactivity to the 27 kDa band was not apparent with any of the antisera. The 26.5 and 28 kDa proteins were immunoreactive on western blots (Figs. 7–10 and 12) of inhibitory fractions at all stages of purification, and absent from fractions lacking activity, establishing a persistent correlation between the inhibitory activity and these proteins. Moreover, the intensity of western blot reactivity was consistent with the amount of inhibitory activity measured in various fractions (cf. Figs. 4,6 vs. Figs. 7,8).

To confirm the identity of the inhibitor, the partially purified inhibitor was precipitated with protein A-bead complexed antibodies to the 28/26.5 kDa proteins (Fig. 11). Immunoprecipitation abolished inhibitory activity in a concentration-dependent manner, whereas preimmune serum had no effect. SDS–PAGE analysis confirmed precipitation of the 26.5/28 kDa proteins with the antibody-complexed beads and removal of both proteins from the resulting supernatant (not shown).

The relationship between the 26.5 and 28 kDa proteins was further examined by two-dimensional (nonequilibrium pH gel electrophoresis/SDS–PAGE) electrophoresis. As seen in Figure 12, the pI of these proteins are identical and no additional proteins are resolved. Both proteins also eluted from an HPLC chromatofocusing column, with pI 4.8 (not shown). Consistent with this pI, the inhibitory protein was insoluble in buffers at pH 4.5–5.5 (data not shown).

Association of these proteins with the testis was confirmed by Western blotting after organ perfusion to remove blood. The 26.5/28 kDa immunoreactive proteins were undiminished in the perfused testis (data not shown).

DISCUSSION

The major CEH inhibitory activity in the 40–65% ASP of testicular cytosol is associated with unique 28 and 26.5 kDa hydrophobic proteins which have been purified to apparent homogeneity by sequential cation exchange chromatography, anion exchange chromatography, and SDS–PAGE. Homo-

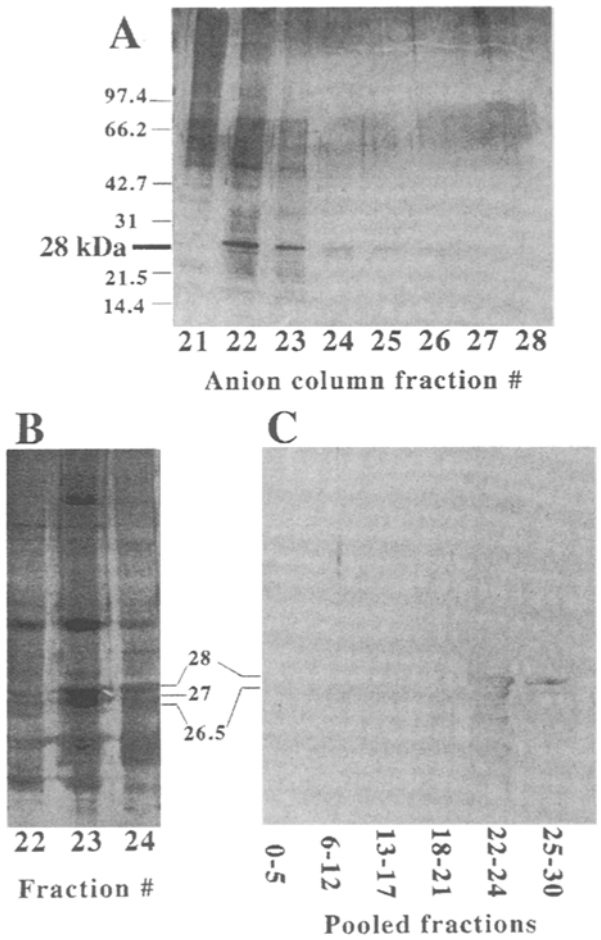


FIG. 8. Analysis of anion exchange fractions by SDS–polyacrylamide gel electrophoresis. Panel A: Silver stained 5–20% SDS–polyacrylamide gradient gel showing prominent 28 kDa band in fractions 22–28, corresponding to elution of inhibitory activity from anion exchange column. Positions of BioRad molecular weight markers are provided for comparison. Panel B: Silver-stained 9–12% SDS–polyacrylamide gradient gel showing resolution of 27 and 26.5 kDa proteins from the major 28 kDa band which co-purified with inhibitory activity (see Fig. 6). Panel C: Western immunoblot of pooled anion exchange fractions showing elution of immunoreactive 28 and 26.5 kDa in fractions 22–24 and 25–30, which contain inhibitory activity (see Fig. 6).

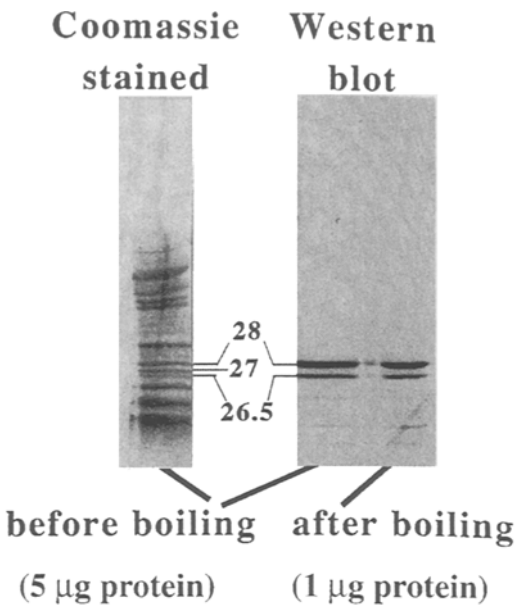


FIG. 9. SDS–polyacrylamide electrophoresis of cation exchange purified CEH inhibitor, showing bands used for immunization and electroelution of purified proteins. Cation exchange fractions 21–24 were pooled and electrophoresed on a 9–12% SDS–polyacrylamide gradient gel. Gels were stained with Coomassie blue to identify 28, 27, and 26.5 kDa bands for excision. Western blot shows immunoreactivity with antibody to 26.5/28 kDa proteins, both before and after boiling of pooled cation exchange fractions. See Figure 1 for other abbreviation.

geneity was confirmed by two-dimensional gel electrophoresis. Inasmuch as these proteins copurify with virtually all fractionation methods, share the same pI and elicit cross-reactive antibodies, they may share surface domains and both may be inhibitory. Although neither could be purified in sufficient quantity to test for individual activity, Coomassie staining, western blotting, and quantitative amino acid analysis indicate that the 28 kDa protein (CIP-28) is 3-fold higher in concentration than the 26.5 kDa protein, in inhibitory fractions at all levels of purification. Moreover, inhibitory activity is immunoprecipitated by antibodies to the 28 and 26.5 kDa proteins. Thus, it is likely that the inhibitory activity is predominantly associated with CIP-28.

Specific inhibitory activity toward testicular CEH was much lower and saturated at a lower inhibitor concentration at 32 than at 37°C, possibly reflecting lower susceptibility of the temperature-labile CEH, which contributes most of the activity at 32°C (39,40). However, this may also reflect the higher concentration of active enzymes at 32°C, whereas only the temperature-stable enzyme is active at 37°C. In support of this view, additional experiments with pancreatic CEH have shown that both the I_{50} and maximum inhibition (I_{max}) decrease as the CEH concentration increases (unpublished data). Inhibition of pancreatic CEH was not different at 32 and 37°C (Fig. 1B), ruling out an intrinsic temperature effect on inhibitory activity.

Although inhibitory activity is stable, even after boiling (Figs. 2A and 9), recoveries are unacceptably low in more highly purified preparations, apparently due to aggregation and adherence to columns, filters, and vessels. This problem was partially solved by excising individual bands from polyacrylamide gels and electroblots for immunization and amino acid analysis, respectively. Although this property prevented most studies with the pure protein in solution under conditions appropriate for CEH activity, it is consistent with a role in regulating the activity of an enzyme which acts at the hydrophilic/hydrophobic interface.

Inasmuch as CIP-28 is hydrophobic and loses its solubility as its homogeneity increases during purification, this protein is apparently stabilized in the native state by the presence of other cellular components, perhaps by association with other cytosolic proteins and with the substrate itself. This is consis-

TABLE 2
Amino Acid Compositions (mol/mol protein) of CEH Inhibitory Proteins and Other Proteins of Similar Molecular Weight

Amino acid	Rat testicular proteins			Rat serum Apo-AI (27 kDa)[a]	Estrogen-regulated protein (28 kDa)[b]	PLAP (28 kDa)[c]
	28 kDa	26.5 kDa	27 kDa			
Asx[d]	17.1	18.4	30.0	31	16.2	22
Glx[e]	18.8	24.8	34.2	40	30.7	23
Ser	27.3	19.1	13.9	8	22.6	36
His	1.9	22.0	5.0	6	6.3	11
Gly	23.0	5.8	13.1	10	20.5	20
Thr	14.0	10.7	11.1	11	15.8	32
Ala	34.5	29.2	25.8	17	22.1	19
Arg	18.3	10.5	10.3	14	21.4	16
Tyr	3.2	3.1	2.3	5	4.0	7
Val	15.7	14.4	19.2	13	13.9	18
Met	3.0	1.8	0.0	8	2.3	7
Phe	5.3	6.9	9.8	6	9.2	10
Ile	6.2	7.6	10.4	4	9.6	26
Leu	23.1	20.2	31.3	28	22.9	32
Lys	36.9	21.6	19.4	21	11.4	13
Pro	16.8	19.8	18.8	7	22.9	12
Trp	ND	ND	ND	6	ND	7
Cys	ND	ND	ND	0	1.4	14

[a]Amino acid composition translated from cDNA sequence (Ref. 33).
[b]Amino acid composition of MCF-7 Estrogen-regulated breast cancer protein (37). Monoclonal antibody to this protein recognizes a 28 kDa cytosolic estrogen-regulated rat Leydig cell protein postulated to be responsible for the early steroidogenic lesion occurring during gonadotropin desensitization (Refs. 35,36).
[c]Amino acid composition translated from cDNA sequence of phospholipase A_2-activating protein from $BC3H_1$ murine smooth muscle cells (Ref. 34).
[d]Asp + Asn residues.
[e]Glu + Gln residues; PLAP, phospholipase-activating protein. See Table 1 for other abbreviation.

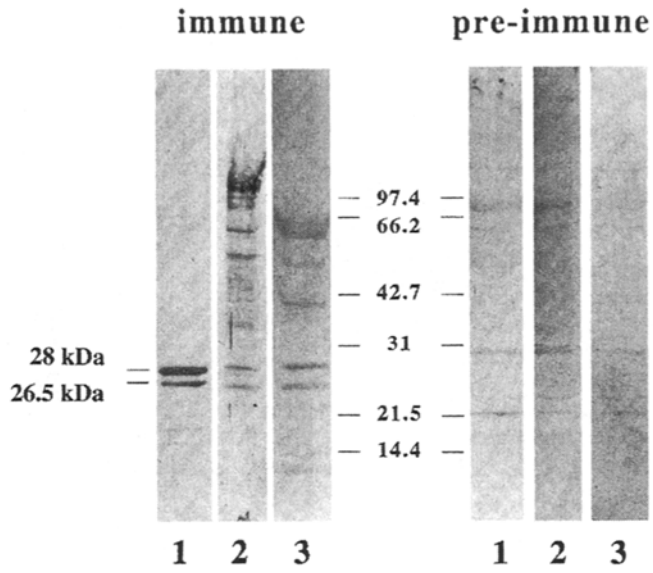


FIG. 10. Specificities of polyclonal antibodies raised to 28 kDa and 26.5 kDa proteins combined (lanes 1), the 26.5 kDa protein alone (lanes 2) or the 28 kDa protein alone (lanes 3). Each lane contains 5 μg cation exchange purified protein (fractions 21–24) electrophoresed on a 9–12% SDS–polyacrylamide gradient gel, transblotted to polyvinylidene difluoride membrane and probed with equivalent amounts of immune or pre-immune IgG. Specific 28 and 26.5 kDa immunoreactive bands are located relative to BioRad (Melville, NY) molecular weight markers.

tent with a role in regulation of CEH by reversible association with the substrate surface. Indeed, a substantial fraction of this protein is recovered from the floating fat layer after differential centrifugation (data not shown). This interaction may alter CEH activity by occluding the surface or displacing bound CEH. Although this mechanism lacks the specificity of classical competitive inhibition, it is consistent with the broad substrate specificities of hydrolytic enzymes and may play an important role in their regulation. Evidence will be presented elsewhere that this protein does in fact inhibit CEH by this mechanism.

CIP-28 can be distinguished from other reported CEH inhibitors, which are heat-labile and soluble in aqueous solution at pH 5–5.2 (13–16,43). The monomeric molecular weight of CIP-28 is much lower than those reported for the other inhibitors (typically, ≈50 kDa). Like the other inhibitors, CIP-28 is highly hydrophobic and elutes diffusely from gel permeation columns. CIP-28 shares similar properties with apolipoprotein A-I (43), including heat stability, molecular weight, and ability to inhibit CEH activity *in vitro*. However, CIP-28 and apoA-I have different amino acid compositions (Table 2), and polyclonal antibodies to CIP-28 do not recognize rat apoA-I on western blots (data not shown). CIP-28 also exhibited important differences in amino acid composition from other surface-active proteins, including PLAP (34) and estrogen-regulated protein (35,36). Thus, CIP-28 appears to be a unique protein.

The physical properties of CIP-28 which hinder recovery also make it difficult to determine the total cellular content. However, a minimum testicular content can be estimated from the amino acid analysis. Correcting for dilution factors, the lower limiting concentrations of the 28, 26.5, and 27 kDa proteins are 8.1×10^{-9}, 2.6×10^{-9}, and 2.2×10^{-9} mols/L, respectively. Although losses during purification would dictate large positive correction factors to obtain accurate values, the calculated values are comparable to minimum effective inhibitor concentrations seen in these studies (~10^{-9} M; see Fig. 5C). Moreover, the activation of CEH seen at very low concentrations (see Fig. 5), which has been reproduced with electroeluted CIP-28 (not shown), suggests that this protein may also be a positive modulator of CEH at concentrations too low to produce inhibition. Clarification of the physiological role of CIP-28 would require knowledge of localized concentrations and interactions with other cellular components. Nevertheless, it is clear that this protein is present in the testis at sufficient levels to be a potentially important regulator of CEH activity.

ACKNOWLEDGMENT

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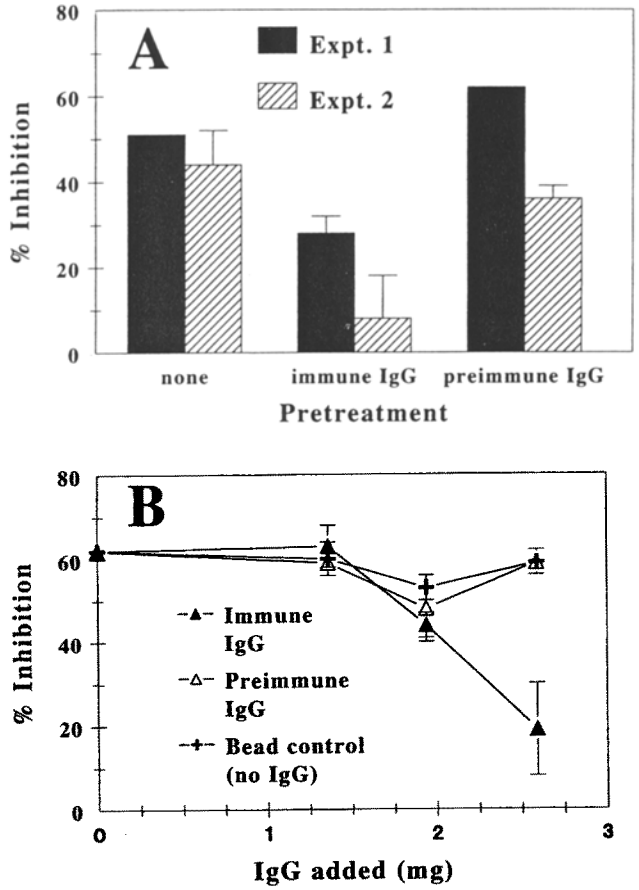


FIG. 11. Concentration-dependent immunoprecipitation of CEH inhibitory activity by immune IgG complexed with protein A conjugated polyacrylic beads. Pancreatic CEH was assayed with addition of equivalent volumes of untreated cation or anion exchange purified inhibitor; supernatant of cation or anion exchange purified inhibitor after precipitation with immune IgG; supernatant of cation or anion exchange purified inhibitor after precipitation with preimmune IgG; or supernatant of cation or anion exchange purified inhibitor after precipitation with protein A conjugated beads alone. (A) Experiment 1: complexes of 2.6 mg preimmune or immune IgG with 22 mg protein A beads were incubated with 2 μg anion exchange purified protein. Experiment 2: complexes of 3 mg preimmune or immune IgG with 25 mg protein A beads were incubated with 3 μg anion exchange purified protein. (B) Inhibitory activity in 8 μg cation exchange purified protein was precipitated by increasing amounts of IgG, as indicated. Values are mean percentage inhibition ± SEM (n = 3). Error bars not seen are contained within bars or symbols. See Figure 1 for other abbreviation.

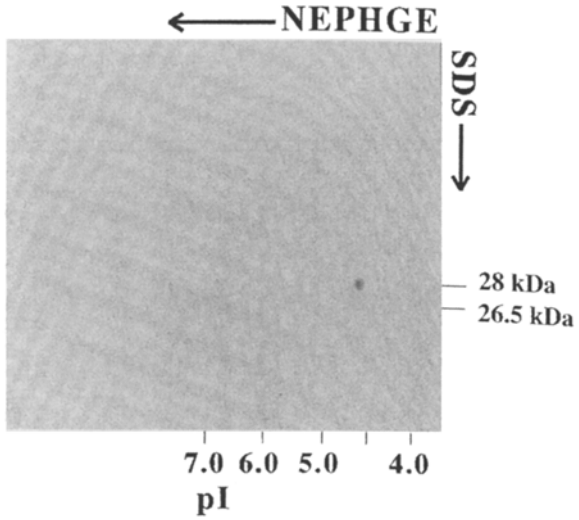


FIG. 12. Western immunoblot from two-dimensional gel electrophoresis of cation exchange purified inhibitor showing mobility of 28 and 26.5 kDa proteins. 250 μg pooled cation exchange fractions 21–28 was separated in the first dimension by nonequilibrium pH gel electrophoresis and in the second dimension on a 10% SDS–polyacrylamide gel, transblotted to polyvinylidene difluoride membrane and probed with 0.8 mg immune IgG. Molecular weight and pI were determined by comparison to two-dimensional molecular weight markers (pI 3.8–7.6; 17–89 kDa) blotted to polyvinylidene difluoride membrane and stained with Coomassie blue.

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Protection by Different Agents Against Inactivation of Lipoxygenase by Hydrogen Peroxide

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ABSTRACT: H_2O_2 is a potent inactivator of lipoxygenase. In this paper, the ability of different agents [mannitol, oleic, stearic and linoleic acid, *n*-butanol, and hydroperoxy octadecadienoic acid (HPOD)] to prevent the inactivation of tomato lipoxygenase by hydrogen peroxide has been studied. The involvement of $OH^{\cdot}$ in the inactivation process is suggested by the ability of mannitol to prevent the loss of activity. This radical would be produced by reaction of H_2O_2 with the Fe(II) lipoxygenase. The most effective protection was displayed by HPOD, the product of the reaction of lipoxygenase with linoleic acid. This result could be explained by the conversion of the native enzyme into the Fe(III) lipoxygenase in the presence of HPOD; the Fe(III) enzyme is not able to react with H_2O_2, and no $OH^{\cdot}$ will be produced. The protective effect obtained with oleic and stearic acid could be explained by an occupation of the active center by these inhibitors. The enzyme would not transform them, but their presence would hamper the conversion of H_2O_2 in $OH^{\cdot}$ and limit the damage in the active center.

Lipids 31, 1245–1250 (1996).

Lipoxygenases (LOX) (EC 1.13.11.12) catalyze the dioxygenation of fatty acids which contain one or more 1(*Z*), 4(*Z*)-pentadiene systems, yielding chiral (*E,Z*) conjugated hydroperoxy fatty acids (1). In mammalian tissues, LOX are involved in the initial steps of the biosynthesis of physiologically active compounds such as leukotrienes and lipoxins (2). The metabolic role of LOX in plants is still uncertain, although they have been implicated in plant senescence, in the response to pest attack as well as wounding (3,4) and in the formation of volatile flavor compounds in many plant food products (5,6).

The presence of one non-heme iron per enzyme molecule has been demonstrated in a number of LOX (7–9). From dormant soybean seeds, the enzyme is isolated as an EPR-silent Fe(II) form which is converted into the active Fe(III) form by the product hydroperoxide (10). The ability of other hydroperoxides such as H_2O_2 to convert the Fe(II)-LOX into the Fe(III) form has been studied by different groups. Some reports suggest that H_2O_2 does not activate the native enzyme (11,12), whereas other authors have demonstrated the ability of H_2O_2 to convert the soybean and reticulocyte 15-LOX from the Fe(II) into Fe(III) form (13,14). Low concentrations of H_2O_2 have been shown to stimulate the dioxygenase activity of soybean LOX (15) and 5-LOX from mammalian cells (16). H_2O_2 is also able to increase the hydroperoxidase activity of LOX, and it can replace lipid hydroperoxide in the oxidation of xenobiotics (17–19).

The irreversible inactivation of soybean LOX in the presence of H_2O_2 was reported in 1967 by Mitsuda *et al.* (20). The sensitivity of LOX to this agent is dependent on the degree of purification, the purified enzymes being more prone to inactivation by H_2O_2 than crude preparations (21). Kazeniac and Hall (22) and Jadhav *et al.* (23) reported the inhibition by H_2O_2 of the development of *cis*-3-hexenal and hexanal in blended tomatoes and in cell free tomato extracts. This data led them to suppose that LOX is involved in the sequence of reactions leading to hexanal and *cis*-3-hexenal formation from unsaturated fatty acids. Despite the fact that enzyme denaturation by H_2O_2 is not specific for LOX (17,24), the inhibitory effect of H_2O_2 has been taken as proof for the involvement of LOX in the flavor formation.

To gain further insight into the effect of hydroperoxides on LOX catalysis, we have studied the inactivation of tomato LOX by H_2O_2 and the influence of substrate, product, and inhibitors on the regulation of this process.

MATERIALS AND METHODS

Tomato LOX was purified using a modification of the method of Bonnet and Crouzet (25), (Suurmeijer, C.N.S.P., and Pérez-Gilabert, M., unpublished results). The specific activity of the enzyme was 10 enzymatic units per mg of protein. SDS-PAGE stained with Coomasie Blue showed three bands in addition to the corresponding to LOX.

Linoleic acid was purchased from Fluka (Bornem, Belgium); *n*-butanol was from Merck (Darmstadt, Germany); H_2O_2 30% wt was obtained from Aldrich (Bornem, Belgium); mannitol, stearic, and oleic acids were from Sigma (Bornem, Belgium). All other chemicals used were of analytical grade.

A H_2O_2 solution was freshly prepared every day, and its concentration was calculated using $\varepsilon_{240} = 39.4\ M^{-1}cm^{-1}$ (26).

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Abbreviations: HPOD, hydroperoxy octadecadienoic acid; LOX, lipoxygenase.

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Oleic, stearic, and linoleic acid were dissolved in methanol. The final concentration of methanol in the reaction medium was 1% (vol/vol).

9-hydroperoxy octadecadienoic acid (HPOD) was prepared by incubation of linoleic acid with tomato LOX in 0.1 M phosphate buffer pH 6.8 at 4°C and under constant aeration. The solution was applied to a 6 mL disposable C-18 extraction column (J.T. Baker Inc., Deventer, The Netherlands) and the product eluted with methanol. High-performance liquid chromatography (HPLC) (27) showed that the main product was 9-HPOD. 13-HPOD was prepared by incubation of linoleic acid with LOX-1 from soybeans in 0.1 M sodium borate buffer pH 10 and purified using the same procedure described for 9-HPOD.

LOX activity was determined spectrophotometrically at 234 nm [ε_{234} = 25,000 $M^{-1}cm^{-1}$, (28)] in a HP8452A diode array at 25°C. The rate was calculated from the linear zone of the product accumulation curve after the lag period.

The inactivation of the enzyme by hydrogen peroxide was confirmed determining the rate of oxygen consumption at 25°C. The equipment was the Hansatech CB1 Oxygen Electrode Unit equipped with magnetic stirrer and connected to a Kratos BD 40 recorder (Hansatech Limited, Norfolk, United Kingdom).

Unless stated otherwise, the reaction medium contained 0.1 M phosphate buffer pH 6.8, 18 µM linoleic acid and 0.016 enzyme units of tomato LOX in a final volume of 1 mL, these being the optimal conditions (Suurmeijer, C.N.S.P., and Pérez-Gilabert, M., unpublished results). One enzymatic unit is defined as the amount of enzyme that gives rise to the appearance of 1 µmol of conjugated diene per minute at 25°C.

In specific experiments, the Fe (III) LOX was obtained by preincubation of the native enzyme with an 80-fold excess of 9-HPOD for 20 min. The excess of hydroperoxide was eliminated by repeated centrifugations (at least four times) of the incubation medium through a Centriprep-30 concentrator (Amicon, IJsel, The Netherlands) as described in (29). The amount of HPOD introduced in the reaction medium along with the Fe(III) LOX was calculated to be less than $5 \cdot 10^{-5}$ µmoles.

RESULTS

The effect of H_2O_2 on the LOX activity was studied following the accumulation of HPOD in the presence of 1.5 mM H_2O_2 (Fig. 1A) and the oxygen consumption (Fig. 1B). The experiment was carried out in two different ways: (a) starting the reaction by addition of enzyme or by addition of substrate (b). Compared with a control without H_2O_2 (curve c), the presence of linoleic acid in the reaction medium prevents the loss of 22% of the activity. In order to exclude that this behavior is the result of a low sensitivity of tomato LOX against the action of H_2O_2, the enzyme was incubated with H_2O_2 at concentrations ranging from 50 to 150 µM (Fig. 2). Samples of the incubation media were taken at different times, and the residual activity was measured by the addition of linoleic acid to the cuvette. In Figure 2, it is shown that when the substrate

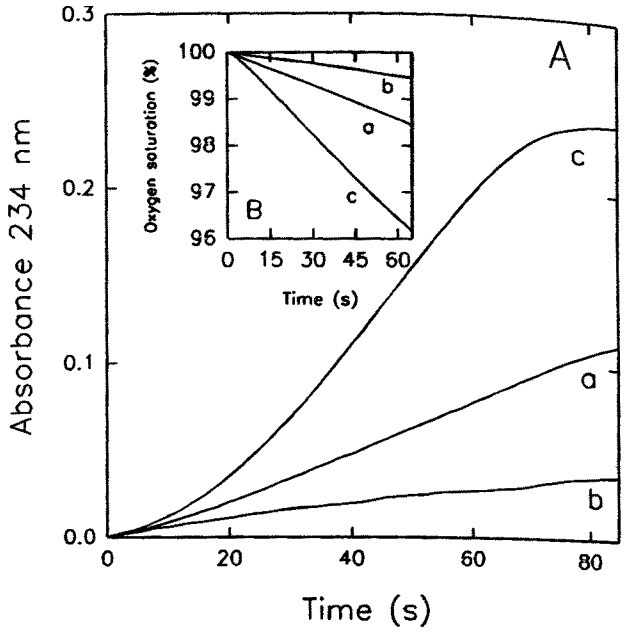


FIG. 1. (A) Progress curves for the oxidation of linoleic acid by tomato lipoxygenase in the standard reaction medium plus 1.5 mM H_2O_2 (traces a and b). The reaction started by the addition of the enzyme (a), or by the addition of the substrate (b). A control without H_2O_2 is shown in trace c. (B) The same experiment was repeated following the rate of O_2 consumption.

is not present in the incubation medium, the inactivation of LOX by H_2O_2 is dependent on the concentration of H_2O_2 and on the duration of the incubation. This inactivation is similar

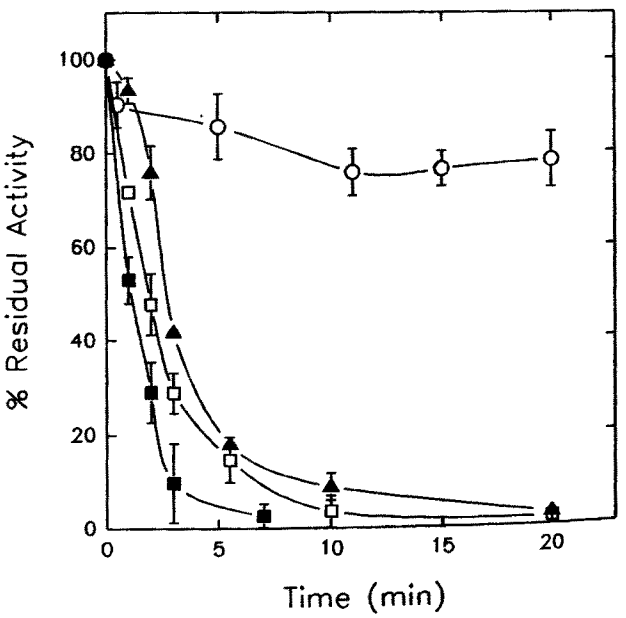


FIG. 2. Inactivation of tomato lipoxygenase by incubation with different concentrations of H_2O_2. The enzyme was incubated in the standard reaction medium containing: no H_2O_2 (○); H_2O_2 50 µM (▲); 75 µM (□), and 150 µM (■). The reaction started by the addition of linoleic acid. The mean value of three determinations ± σ_n is represented.

to that displayed by soybean LOX (20) and indicates that the sensitivity of both enzymes toward H_2O_2 is similar.

The results presented in Figures 1 and 2 suggest that the presence of linoleic acid somehow prevents the inactivation of tomato LOX by a high concentration of H_2O_2. This observation could be explained either by a direct effect of the substrate occupying the active center and thus preventing the attack of H_2O_2 on the catalytic site or by an effect of the product of the reaction.

To check if there was any relationship between the linoleic acid concentration and the percentage of the residual activity, the initial rate was measured in the presence of different concentrations of H_2O_2. The results were similar when different linoleic acid concentrations, i.e., 2.5 μM, 18 μM, or 36 μM were used (Fig. 3).

In order to find out if the product of the reaction played a role in the mechanism of protection, the enzyme was incubated with 2.5 μM 9-HPOD or 2.5 μM 13-HPOD, plus 50 μM H_2O_2 and the residual activity at different intervals of time was measured. From Figure 4, it can be concluded that the presence of either 13-HPOD (◆) or 9-HPOD (△) indeed protects the enzyme against denaturation.

This effect of HPOD can be explained by a physical protection of the active center or by a difference in the sensitivity of Fe(II)-LOX and Fe(III)-LOX to the action of H_2O_2. To distinguish between these two possibilities, the native Fe(II) LOX was preincubated for 20 min with 9-HPOD, and the excess of HPOD was removed by ultrafiltration (see the Materials and Methods section). The obtained Fe(III) enzyme was incubated with 50 μM H_2O_2, and the residual activity was measured. The results presented in Figure 4 indicate that there is no difference in activity between the experiment carried out with the enzyme preincubated with HPOD (○) or with the enzyme incubated with H_2O_2 + HPOD (◆ and △).

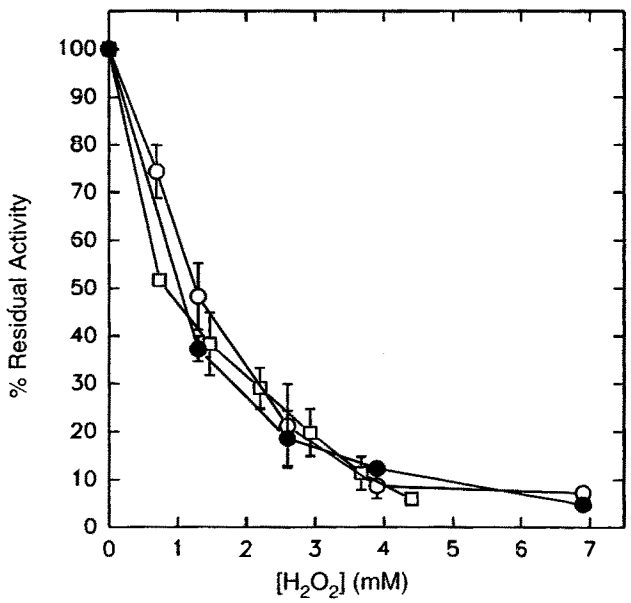


FIG. 3. Effect of the substrate on the inactivation of lipoxygenase by H_2O_2. The reaction medium contains different concentrations of H_2O_2, 0.016 enzymatic units of tomato lipoxygenase and 2.5 μM (□), 18 μM (○), or 36 μM (●) linoleic acid. The reaction started by the addition of lipoxygenase. The mean value of three determinations ± σ_n is represented.

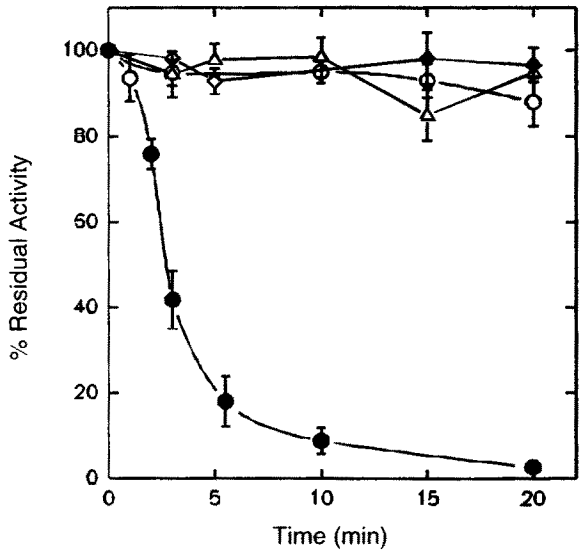


FIG. 4. Effect of HPOD on the inactivation of lipoxygenase by H_2O_2. Fe(II) (●) or Fe(III)-LOX (○) were incubated with 50 μM H_2O_2. Fe(III)-LOX was prepared as described in the Materials and Methods section. In another experiment, lipoxygenase was incubated in the presence of 50 μM H_2O_2 + 2.5 μM 13-HPOD (◆), or 2.5 μM 9-HPOD (△). In the three experiments, the reaction started by the addition of 18 μM linoleic acid. The mean value of three determinations ± σ_n is represented.

Early reports (20,30) described the differences in the protective effect displayed by certain competitive inhibitors against the inactivation of soybean LOX by H_2O_2. They observed that analogs of linoleic acid were able to protect the enzyme, whereas saturated monohydric alcohols were not effective. These differences were explained supposing that the linoleic acid analogs were able to occupy the active center, whereas the alcohols could merely attach to a hydrophobic region of the protein. In the present study, tomato LOX was incubated with H_2O_2 in the presence of different concentrations of stearic, oleic acid, or *n*-butanol (Fig. 5). It was found that oleic and stearic acids were able to prevent the inactivation when concentrations in the range of 36 μM were tested (no higher concentrations were used to avoid the turbidity of the reaction medium). However, *n*-butanol, even at a concentration of 0.2 M in the incubation medium, was ineffective in preventing the enzyme denaturation by H_2O_2 (Fig. 5). In the presence of 0.2M *n*-butanol, the residual activity is 58% with respect to a control without *n*-butanol.

It has been suggested (31) that the inactivation produced by H_2O_2 may be a consequence of reactive oxygen species such as $OH^{\cdot}$ produced by reaction with the Fe(II) in the enzyme. This radical may modify different amino acids in the active center of the enzyme such as histidine (20). The effect of mannitol, a well-known radical scavenger, (32) was also

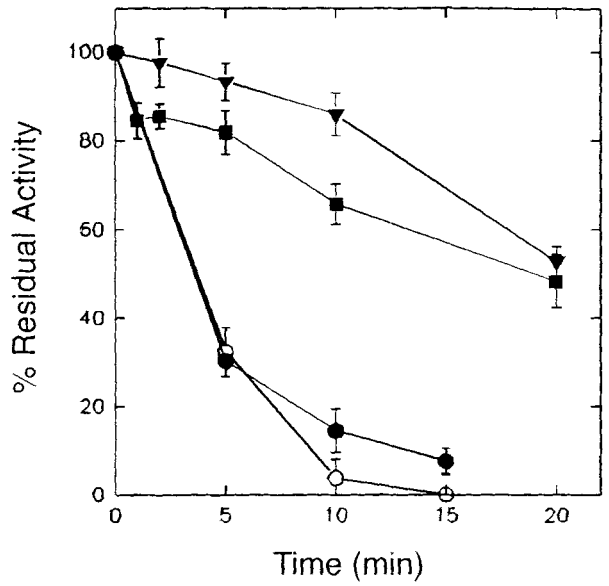


FIG. 5. Effect of different lipoxygenase inhibitors. The enzyme was incubated with 50 μM H_2O_2 plus 36 μM oleic acid (▼); 36 μM stearic acid (■); *n*-butanol 0.1M (○) or 0.2 M (●). The reaction started by the addition of 18 μM linoleic acid. The mean value of three determinations ± σ_n is represented.

tested, and it was found that only concentrations in the range of 0.2 M were able to partially prevent the inactivation (Fig. 6).

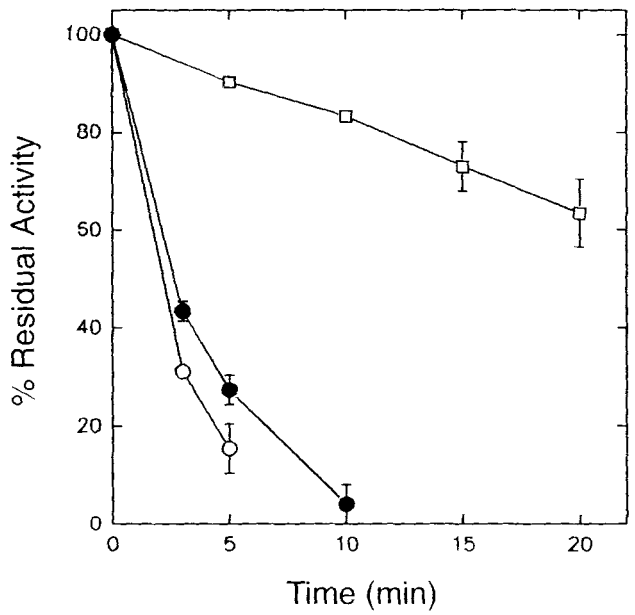


FIG. 6. Effect of mannitol. Lipoxygenase was incubated in the standard reaction medium with 50 μM H_2O_2 plus different concentrations of mannitol: 0.2 mM (○); 4 mM (●), and 0.2 M (□). The reaction started by the addition of 18 μM linoleic acid. The mean value of three determinations ± σ_n is represented.

DISCUSSION

The protective effect of different substrates on the hydroperoxidase activity of LOX against inactivation by H_2O_2 has been described recently (17–19). In the absence of a hydrogen donor, H_2O_2 causes a decrease in the rate of xenobiotic oxidation. In this paper we describe for the first time the protection of the dioxygenase activity of LOX by the substrate of the reaction.

The presence of linoleic acid in the reaction medium strongly prevents the inactivation of lipoxygenase by high concentrations of H_2O_2. In Figure 1A the progress curves for the oxidation of linoleic acid by LOX in the presence of 1.5 mM H_2O_2 are shown, thus indicating that the initial velocity depends on the order of addition of the reagents, namely first the substrate (trace a) or first the enzyme (trace b). When the reaction is started by the addition of substrate, the enzyme loses 87% of its initial activity in the time lapse between the addition of the enzyme and the addition of linoleic acid (about 10 s). However, when the reaction starts by the addition of LOX, the inactivation is 65%. The same results were obtained when the reaction was determined polarographically as the rate of O_2 consumption (Fig. 1B), thus confirming the inactivation of the enzyme.

When tomato LOX is preincubated with different concentrations of H_2O_2, a time-dependent inactivation is observed (Fig. 2). A concentration of 150 μM H_2O_2 abolishes the enzymatic activity in less than 8 min. This result indicates that the sensitivity of tomato LOX to hydrogen peroxide is similar to that reported by Mitsuda *et al.* (20) and Egmond *et al.* (31) for soybean lipoxygenase.

If the decrease in the inactivation shown in Figure 1 is a direct result of the presence of linoleic acid in the reaction medium, different responses should be expected depending on the concentration of the substrate. However, the results presented in Figure 3 show that the residual activity measured in the presence of different amounts of H_2O_2 is the same regardless of the linoleic acid concentration used (2.5, 18, or 36 μM). An alternative explanation is that the product of the reaction was the "real" responsible for the protection displayed by linoleic acid. To check this possibility, LOX was preincubated with 50 μM H_2O_2 and 2.5 μM 9-HPOD, the product of the tomato LOX reaction, or with 13-HPOD. The results presented in Figure 4 indicate that both 9- and 13-HPOD are able to avoid partially the inactivation of the enzyme. Egmond *et al.* (31) and Aoshima (33) reported a correlation between the fluorescence quenching and the inactivation of soybean LOX by H_2O_2. This quenching would be due to the conversion of Fe(II)-LOX into the Fe(III)-LOX (31):

$$\text{LOX-Fe(II)} + H_2O_2 \rightarrow \text{LOX-Fe(III)} + OH^{\cdot} + OH^{-} \quad [1]$$

The active oxygen species produced in this process would react with amino acids near or at the active site (34) inactivating the enzyme. Thus, the protection of LOX by HPOD (Fig. 4) could be explained by the conversion of the native enzyme in the Fe(III) LOX which, according to the above

scheme, is not able to react with H_2O_2. To test this hypothesis and to rule out a protection due to the attachment of the HPOD to the active center, the residual activity of Fe(III) enzyme after its incubation with 50 μM H_2O_2 was measured. In Figure 4 it is shown that while the native enzyme completely loses its activity after 20 min of incubation with H_2O_2 the Fe(III)-LOX retains its activity. This larger sensitivity of the Fe(II) toward H_2O_2 has been observed by Percival *et al.* (21) with purified human 5-LOX.

The model proposed in reaction 1 cannot explain the results obtained in this work with competitive inhibitors such as oleic and stearic acid (Fig. 5). In a recent paper, Van der Heijdt *et al.* (35) suggested the possibility that oleic acid would bind to the same site on LOX as linoleate. In this sense the protective effect obtained with oleic and stearic acid could be explained by an occupation of the active center by these inhibitors. The enzyme would not transform them, but their presence would hamper the conversion of H_2O_2 in $OH^{\cdot}$ and limit the damage in the active center. The effect of *n*-butanol, another competitive inhibitor of LOX, was also studied, but concentrations up to 0.2 M of this compound were unable to prevent the inactivation of tomato LOX by H_2O_2 (Fig. 5). This result is in accordance with the data reported for the soybean enzyme (30,36) and have been interpreted supposing that these alcohols attach to a site other than the catalytic site (30). Boyington *et al.* (37) described the existence of three hydrophobic cavities in LOX-1 from soybean; two of them connected to the iron site, while the largest cavity is not in contact with the active center. Though the three-dimensional structure of tomato LOX has not been established up until now, our results suggest the existence of similarities between tomato and soybean LOX.

The involvement of $OH^{\cdot}$ in the inactivation process suggested in reaction 1 is supported by the ability of mannitol to protect the enzyme from inactivation. In Figure 6, the results obtained by adding different amounts of mannitol to the incubation medium are shown. The high concentration of mannitol (0.2 M) needed to protect the enzyme against inactivation is explained considering that, in order to scavenge this $OH^{\cdot}$, the mannitol molecules must be present in the active center at the same time as H_2O_2, but the hydrophobicity of the active center makes the access of mannitol difficult. *In vivo*, an increase in the production of H_2O_2 has been observed after the addition of elicitors to plant cell cultures (38). This increase precedes LOX elicitation (39), suggesting that this enzyme may contribute to diminish the concentration of H_2O_2 at this metabolic stage.

The results in this paper suggest that the protective effect displayed by linoleic acid against the inactivation of tomato LOX by H_2O_2 may be the result of two different processes, one physical and the other chemical. The physical protection would be a consequence of the occupation of the active center by the substrate which would hinder the access of H_2O_2 to the catalytic site. This effect can be mimicked by those competitive inhibitors of LOX that attach to the active center. The chemical protection is produced by the HPOD synthesized by LOX or present in linoleic acid, and consists of the conversion of Fe(II)-LOX into the Fe(III) form which cannot catalyze reaction 1 and thus is insensitive to H_2O_2. Radical scavengers diminish LOX inactivation only if a high concentration is used. The lower sensitivity of crude extracts of LOX to H_2O_2 compared with purified enzyme preparations (21) could be explained by the presence in the extraction medium of "protective agents" such as substrates, competitive inhibitors, or radical scavengers that would be subsequently removed during the purification. The better understanding of the mechanism of LOX inactivation by H_2O_2, also may facilitate optimization of purification procedures.

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Lipid Peroxidation in Low Density Lipoproteins from Human Plasma and Egg Yolk Promotes Accumulation of 1-Acyl Analogues of Platelet-Activating Factor-Like Lipids

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ABSTRACT: Oxidative modification of low density lipoprotein (LDL) is known to be a key event for induction of atherosclerosis. However, there has been little progress in structural elucidation of oxidized lipids, especially oxidatively fragmented phospholipids retaining a glycerol backbone. In this study, we found that LDL derived from egg yolk has no platelet-activating factor (PAF) acetylhydrolase activity, and that prolonged incubation of egg yolk LDL with Cu^{2+} resulted in the formation of various PAF-like lipids: 1-acyl type phosphatidylcholines with an *sn*-2-short-chain dicarboxylate or monocarboxylate group. Only a very small amount of the PAF-like lipid having an *sn*-2-short-chain monocarboxylate group was detected by gas chromatography–mass spectrometry in Cu^{2+}-oxidized LDL from human plasma with high PAF-acetylhydrolase activity, which has been reported to hydrolyze PAF-like lipids to lysophosphatidylcholines. Preincubation of plasma LDL with diisopropyl fluorophosphate dose-dependently inhibited PAF-acetylhydrolase activity, resulting in accumulation of the PAF-like lipids when the LDL was oxidized with Cu^{2+}. As well as PAF and lysophosphatidylcholines, several PAF-like lipids were found to inhibit [^{3}H]thymidine incorporation into cultured vascular smooth muscle cells derived from rat aorta. The possible formation of PAF-like lipids by lipid peroxidation in LDL is discussed as well as its possible significance for induction of atherosclerosis.

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Platelet-activating factor (PAF) is a phospholipid autacoid with various potent biological activities in proinflammatory cells (1), but both its extracellular and intracellular levels are strictly controlled by enzymes for its synthesis and degradation (2). One structural feature of PAF is its glyceryl ether skeleton, which is resistant to hydrolytic enzymes and important for high affinity binding to its receptors. Another feature is a short-chain acyl group attached at the *sn*-2-position to the glycerol backbone of the PAF molecule. All natural PAF identified so far in animal tissues, body fluids, and cells have an *sn*-2-acetyl residue (3), except a PAF fraction purified from a lipid extract of the earthworm (*Pheretima* sp.), which is abundant in 1-*O*-alkyl-2-propionyl-*sn*-glycero-3-phosphocholine (GPC) (4). The earthworm was suggested to utilize more propionyl-CoA than acetyl-CoA for PAF synthesis (4). Noteworthy in this connection are the identifications of various 1-long-chain acyl analogues of PAF (acylPAF) and its analogues having an *sn*-1-long-chain acyl group and *sn*-2-short-chain dicarboxylate (DC), ω-hydroxymonocarboxylate, or monocarboxylate (MC) group other than an acetyl group, in a lipid extract from bovine brain (5–7). The possible formations of these unique phosphatidylcholines (PC) having an *sn*-2-short-chain acyl group during peroxidation of PC with an *sn*-2-polyunsaturated acyl group was extensively examined in an *in vitro* experiment with PC liposomes incubated with Fe^{2+}/EDTA in the presence of ascorbic acid (8,9). Results showed that an additional series of PC having an *sn*-2-DC semialdehyde residue were generated by lipid peroxidation as well as these three types of PAF-like lipids detected in the bovine brain lipid extract. Interestingly, the chain length of the *sn*-2-acyl group in the PAF-like lipids was dependent on the position of a double bond in the polyunsaturated fatty acyl moiety vicinal to the ester linkage in the parent PC molecule. Thus, the oxidative degradation of PC with a docosahexaenoate residue would lead to the generation of PC having both a propionate residue and a succinate residue, which were MC–PC and DC–PC, respectively, with an *sn*-2-acyl group with the shortest chain length among the authentic PC tested. A DC–PC having an azelaoyl group was previously reported to be produced by the oxidation of PC with oxyhemoglobin, and to be cytotoxic to erythrocytes (10). Oxidatively degraded PAF-like lipids were also found to be gener-

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Abbreviations: acylPAF, 1 long-chain acyl analogue of platelet- activating factor; BSA, bovine serum albumin; DC, short-chain dicarboxylate; DFP, diisopropyl fluorophosphate; FAB–MS, fast atom bombardment–mass spectrometry; GC–MS, gas chromatography–mass spectrometry; GLC, gas–liquid chromatography; GPC, *sn*-glycero-3-phosphocholine; LDL, low density lipoprotein; lysoPAF, lysoplatelet-activating factor; lysoPC, lysophosphatidylcholine; MC, short-chain monocarboxylate; PAF, platelet-activating factor; PAF–AH, platelet-activating factor acetylhydrolase; PC, phosphatidylcholine; *t*BDMS, *tert*-butyldimethylsilyl; TLC, thin-layer chromatography; VSMC, vascular smooth muscle cells.

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ated from membrane phospholipids in cultured vascular endothelial cells exposed to *tert*-butylhydroperoxide or cumen hydroperoxide (11).

Oxidized low density lipoprotein (LDL) is known to be a key substance for induction of atherosclerosis. LDL oxidized *in vitro* has been found to have various biological effects such as induction of chemotaxis of monocytes and promotion of transformation of macrophages to foam cells (12). Although some oxidation products of phospholipid components other than lysophosphatidylcholine (lysoPC) in LDL were suggested to be the active components for these effects, there have been only a limited number of reports on successful chemical characterization of these active lipids (13,14), because complicated reactions usually proceed in parallel during lipid peroxidation, and almost all the oxidation products are unstable. It should be mentioned, however, that Kamido *et al.* (15) have recently identified various PC with a DC semialdehyde residue in copper-oxidized LDL from human plasma by liquid chromatography–mass spectrometry.

An aim of this study was to clarify whether several types of PAF-like lipid are formed in significant amounts as secondary, but relatively stable, oxidation products of PC during incubation of LDL with Cu^{2+}. We also examined the possible cytotoxic effect of PAF-like lipids on cultures of rat aorta vascular smooth muscle cells (VSMC) as model cells at the subendothelium.

MATERIALS AND METHODS

Chemicals. [^{3}H]Thymidine (5.0 Ci/mmol) and 1-*O*-[^{3}H]-octadecyl-*sn*-glycero-3-phosphocholine (GPC, 80–180 Ci/mmol) were purchased from Amersham Japan (Tokyo, Japan). 1,2-Diheptadecanoyl-GPC, 1-heptadecanoyl-2-lyso-GPC, 1-palmitoyl-2-lyso-GPC, *Bacillus cereus* phospholipase C and fatty acid-free bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). Hexadecyl-PAF and its lyso derivative (lysoPAF) were from Bachem Feinchemikalien AG (Bubendorf, Switzerland). *tert*-Butyldimethylchlorosilane was obtained from Sin-Etsu Chemical Co. (Tokyo, Japan). All other chemicals were purchased from Wako Pure Chemical Co. (Osaka, Japan).

PC containing an *sn*-2-short-chain acyl group such as an acetyl, butyl, caproyl, or capryloyl group were prepared by treating 1-palmitoyl-2-lyso-GPC with the corresponding short-chain acid anhydride, as described previously (16). 1-Palmitoyl-2-succinyl-GPC and 1-palmitoyl-2-glutaryl-GPC were obtained by treating 1-palmitoyl-2-lyso-GPC with succinic anhydride and glutaric anhydride, respectively, by the method described previously (6). 1-Palmitoyl-PC containing *sn*-2-azelaoyl group was prepared as described previously (6), except for the use of azelaoyl chloride instead of adipoyl chloride.

Preparation of LDL. Egg yolk-derived LDL was purchased from Q P Co. (Tokyo, Japan). Human LDL was isolated from pooled plasma anticoagulated with citrate–phosphate–dextrose solution from healthy volunteers by near vertical spin discontinuous density ultracentrifugation as described previously (17). LDL protein was determined by the method of Lowry *et al.* (18).

Fractionation of phospholipids. Lipids were extracted from LDL solution by the method of Bligh and Dyer (19). Phospholipids in the extract were fractionated into several classes by thin-layer chromatography (TLC) on a Merck Silica gel 60 plate (0.25 mm-thickness) with a developing solvent system of chloroform/methanol/water (65:35:5, by vol), and located under an ultraviolet lamp after spraying with an 1% ethanolic solution of 6-(*p*-toluidino)-2-naphthalenesulfonic acid. Regions of the silica gel containing individual phospholipids were scraped off the plate, and suspended in 4 mL of water. The phospholipids were recovered by the method of Bligh and Dyer (19), and their lipid–phosphate contents were quantified as described by Chalvarjian and Rudnicki (20).

Fatty acid analysis. PC and lysoPC from human plasma and egg yolk LDL were subjected to methanolysis by suspending the phospholipid in 0.5 mL of a 0.5% methanolic solution of hydrochloride at 100°C for 3 h with 1,2-diheptadecanoyl-GPC and 1-heptadecanoyl-GPC, respectively, as internal standards (21). The reaction mixture was diluted with 3 mL of water, and the fatty acid methyl esters were extracted twice with 3 mL of *n*-hexane. Gas–liquid chromatography (GLC) was done in a Shimadzu GC-15A gas chromatograph equipped with a DB-225 capillary column (30 m × 0.242 mm i.d., 0.25 μm thickness; J&W Scientific). Helium was used as a carrier gas, and the injection and detector temperatures were both set at 250°C. The column temperature was first 120°C for 1 min then increased to 220°C at 10°C/min.

Induction of lipid peroxidation. A solution of human LDL in 0.2 M phosphate buffer, pH 7.4 (0.5–0.6 μmol PC/mL) was preincubated at 37°C for 30 min in the presence or absence of diisopropyl fluorophosphate (DFP). A solution of commercial egg yolk-derived LDL was diluted with 0.2 M phosphate buffer to 0.5 μmol PC/mL, and then preincubated at 37°C for 30 min in the absence of DFP. Lipid peroxidation was induced by incubating the LDL with 35 μM $CuSO_4$ at 37°C for 24 h. The reaction mixture was adjusted to pH 2–3 with 1 M HCl, and the lipids were extracted by the method of Bligh and Dyer (19).

Structural analysis of PAF-like lipids. Phospholipids from oxidized LDL were separated by TLC on a Merck Silica gel 60 plate in a solvent system of chloroform/methanol/28% ammonium hydroxide (65:35:8, by vol). The plate was divided into several zones (PC zone, lysoPC zone, zone between PC and lysoPC, and zone below lysoPC), the silica gel in these zones was scraped off, and the lipids were extracted by the method of Bligh and Dyer (19) after adjusting the aqueous phase to pH 2–3, as described previously (8). Then 1 or 2 μL aliquots of the chloroform/methanol (2:1, vol/vol) solutions of phospholipid extracted from these zones were mixed with 1 μL of thioglycerol on the probe tip of insertion for fast atom bombardment–mass spectrometry (FAB–MS) of a JEOL JMS-SX102A mass spectrometer (Tokyo, Japan). The gun

high voltage, filament current, emission current, and accelerating voltage were 3 kV, 1A, 1 mA, and 10 kV, respectively.

Quantitation of MC- and DC-PC by gas chromatography-mass spectrometry (GC-MS). PAF-like lipids such as MC-PC and DC-PC were quantified by GC-MS with use of 1-palmitoyl-2-deuterated acetyl-GPC and 1-palmitoyl-2-deuterated succinyl-GPC, respectively, essentially as described previously (8). Briefly, oxidized phospholipids fractionated by TLC as described above were hydrolyzed with *Bacillus cereus* phospholipase C, and the resultant glycerides were treated with *tert*-butyldimethylchlorosilane in dimethylformamide in the presence of imidazole at 65°C for 1 h to derivatize its hydroxyl groups. For determination of DC-MC, the *tert*-butyldimethylsilyl (*t*BDMS) derivatives of glycerides were then treated with etherial diazomethane at room temperature for 10 min. The *t*BDMS derivatives of MC-PC and DC-PC were purified by TLC on a Silica gel 60 plate in a solvent system of *n*-hexane/diethyl ether (9:1, vol/vol), and analyzed in a Hitachi M-80 mass spectrometer coupled with a gas chromatograph equipped with a capillary column (J&W Scientific, DB-1, 30 m, 0.25 mm i.d., 0.25 μm-thickness). The column temperature was programmed to rise from 200 to 320°C at a rate of 10°C/min.

Measurement of PAF acetylhydrolase (PAF-AH) activity. Human plasma LDL was pretreated with various concentrations of DFP or vehicle for 30 min. To 200 μL of 0.1% BSA solution containing [^{3}H]PAF (0.02 nmol, 2.5 μCi) was added 4 mL of a solution of native egg yolk or human plasma LDL in 0.2M phosphate buffer pretreated with DFP or vehicle alone. The mixture was incubated at 37°C for various times, and then lipids were extracted by the method of Bligh and Dyer (19), and analyzed by TLC in a solvent system of chloroform/methanol/water (65:35:5, by vol). The silica gel was scraped from various zones of the TLC plate and mixed with 4 mL of Sintisol EX-H (Dojin Chemicals, Kumamoto, Japan) for measurement of the radioactivity in a liquid scintillation counter. The percentage conversions of [^{3}H]PAF to [^{3}H]lysoPAF were then calculated.

Cytotoxic effects of PAF-like lipids. The cytotoxic effects of PAF-like lipids were assessed by measuring inhibition of [^{3}H]thymidine incorporation into DNA in cultured VSMC from rat aorta stimulated with 10 ng/mL of platelet-derived growth factor, as described previously (21). PAF-like lipids were dispersed in Dulbecco's modified Eagle's medium containing 0.1% BSA, and 300 μL aliquots were added to VSMC in a 24-well plate, followed by 100-μL each of solutions of platelet-derived growth factor and [^{3}H]thymidine (0.1 μCi/well).

RESULTS

Chicken serum or plasma is reported to be devoid of PAF-AH activity (22), and therefore LDL derived from hen egg yolk is expected to lack this enzyme also, unlike that from human plasma. The PAF-AH activities of LDL from egg yolk and human plasma were assessed by measuring the conversion rate (mol%) of [^{3}H]PAF to [^{3}H]lysoPAF during incubation with 37 μM Cu^{2+} at 37°C for various times. As predicted, there was no significant degradation of [^{3}H]PAF to [^{3}H]lysoPAF during incubation of egg yolk LDL with Cu^{2+} (data not shown). Human plasma LDL was found to have high PAF-AH activity, and this activity was inhibited dose-dependently by pretreatment of the LDL with 1, 2, 5, 10 mM DFP (data not shown), in harmony with previous reports (14,23,24).

On incubation with Cu^{2+}, the lysoPC content of human plasma LDL increased markedly from 13 ± 3.8 (mean ± SE, n = 4) to 168 ± 11 nmol/mL of LDL solution, consistent with earlier reports on human LDL oxidized by Cu^{2+} (25,26), whereas, that of egg yolk LDL increased only slightly (155 ± 9% of that of native LDL, n = 3). Pretreatment of human plasma LDL with 1 and 5 mM DFP resulted in reduced generation of lysoPC during incubation with Cu^{2+} (91 ± 3.1 and 59 ± 3.0 nmol/mL of LDL solution, respectively, n = 3). These results suggest that oxidized PC including PAF-like lipids accumulate in a significant amount during the incubation with Cu^{2+} of egg yolk LDL and of human plasma LDL pretreated with DFP, since a considerable portion of the PAF-like lipid could escape the hydrolysis by PAF-AH to lysoPC. Therefore, we next tried to identify the PAF-like lipids in these Cu^{2+}-oxidized LDL by MS.

The positive ion FAB-mass spectrum of a fraction containing DC-PC in a lipid extract of oxidized egg yolk LDL separated by TLC included fragment ions characteristic of acyl analogues of PAF-like lipids: $[phosphocholine]^+$ (*m/z* 184), and $[M + H]^+$ (*m/z* 596, 610, 666, and 694 probably for 1-palmitoyl-2-succinyl-, 1-palmitoyl-2-glutaryl-, 1-palmitoyl-2-azelaoyl-, 1-stearoyl-2-azelaoyl species, respectively). The negative ion FAB-mass spectrum of the TLC fraction containing DC-PC supported the existence of 1-palmitoyl- and stearoyl-PC (Fig. 1B). The strong signals at *m/z* 255 and 283 could be assigned to palmitic and stearic anions, respectively. Furthermore, there were characteristic signals such as those of $[M - R_2CO - choline + H]^-$ (*m/z* 391 and 419 for 1-palmitoyl- and stearoyl-species, respectively), $[M - R_2CO - CH_2CH_2N(CH_3)_3 + 2H]^-$ (*m/z* 409 and 427), $[M - R_2CO - N(CH_3)_3]^-$ (*m/z* 435 and 463) and $[M - R_2CO - CH_3 + H]^-$ (*m/z* 480 and 508). Several signals were informative for the existence of DC-PC; these were $[M - H]^-$ (*m/z* 664 and 692 for 1-palmitoyl-2-azelaoyl and1-stearoyl-2-azelaoyl species). Similar negative-ion and positive-ion mass spectra were obtained by FAB-MS of DC-PC fractionated by TLC from a lipid extract of oxidized human plasma LDL pretreated with 1 mM DFP. Its negative-ion FAB-mass spectrum is shown as well as that of authentic 1-palmitoyl-2-azelaoyl-GPC in Figure 1C and A, respectively.

As shown above, FAB-MS was useful for structural elucidation of DC-PC, which are predominant PAF-like lipids in oxidized LDL from human plasma and egg yolk. Next, we used GC-MS for the quantification of not only major DC-PC but also minor PAF-like lipids. The PAF-like lipids generated from oxidized egg yolk LDL were separated by TLC into two fractions for GC-MS, as described in the Materials and Meth-

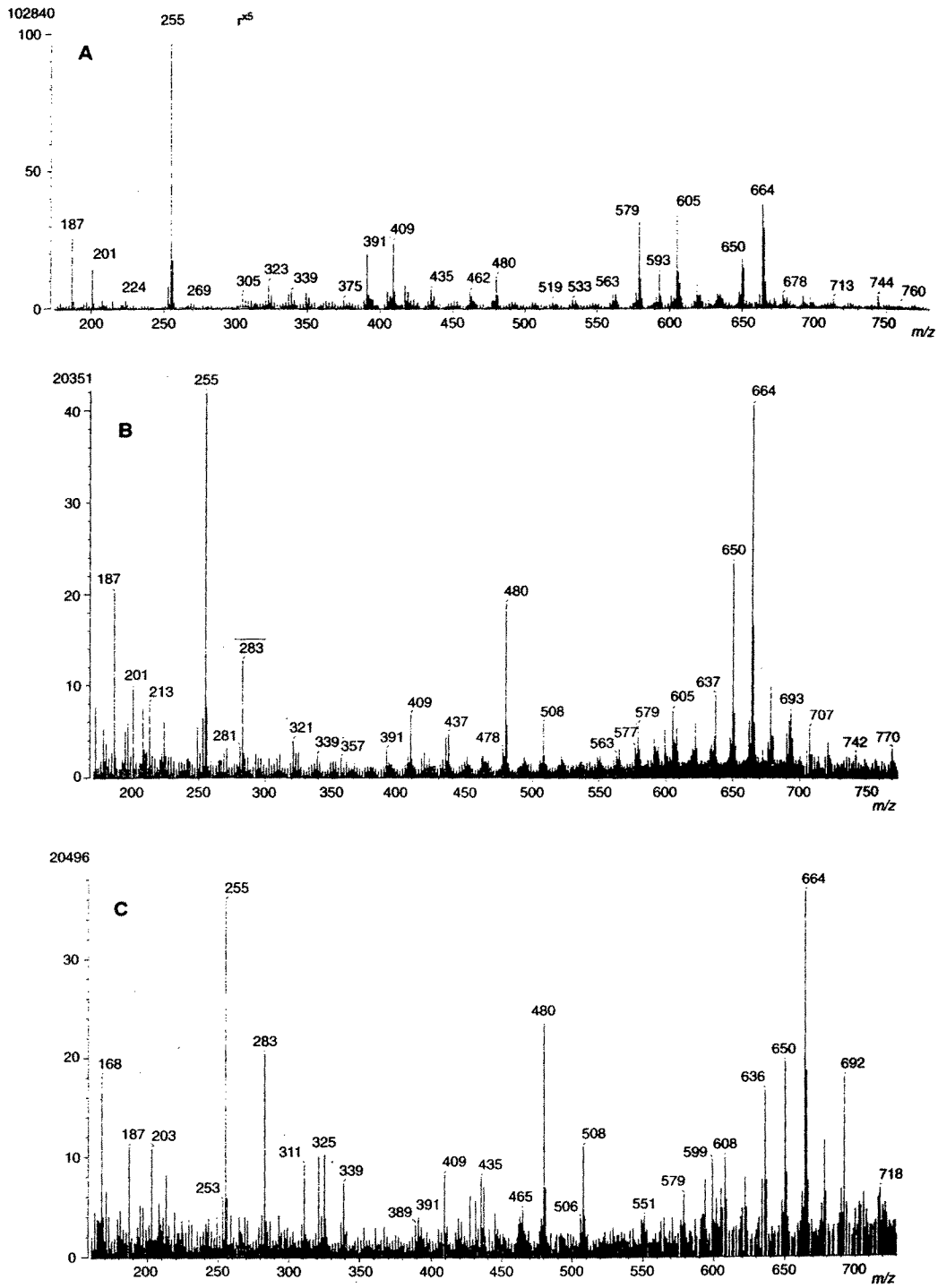


FIG. 1. Negative-ion fast atom bombardment mass spectra of authentic 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine (A), short-chain dicarboxylate–phosphatidylcholine in Cu^{2+}-oxidized low-density lipoprotein from egg yolk (B) and human plasma pretreated with 1 mM diisopropyl fluorophosphate (C).

ods section. Results show that these two fractions consisted of eight species each of MC–PC and DC–PC, respectively. Their contents in the LDL are expressed as nmol/μmol of starting PC in Table 1. The most abundant MC–PC and DC–PC were 1-palmitoyl-2-octanoyl- and 1-palmitoyl-2-azelaoyl-species, respectively, both of which were assumed to be formed during peroxidation of 1-palmitoyl-2-linoleoyl-GPC. The total amount of DC–PC was about 100 times that of MC–PC.

No significant amount of DC–PC was detected in oxidized human plasma LDL, the fatty acid composition of PC in which was as follows: 16:0 (34.3 ± 2.5 mole%, mean ± SD,

TABLE 1
Molecular Species Composition of DC–PC and MC–PC Formed by Oxidative Fragmentation of PC in LDL[a]

PAF-like lipid (*sn*-1/*sn*-2)	Amount as nmol/μmol starting PC		
	Egg yolk LDL	Human plasma LDL	DFP-treated human plasma LDL
DC–PC			
16:0/4:0	0.71 (4.3)	—	—
18:0/4:0	0.19 (1.2)	—	0.22 (3.1)
16:0/5:0	0.73 (4.4)	—	0.57 (8.1)
18:0/5:0	1.06 (6.4)	—	0.68 (9.6)
16:0/8:0	1.72 (10.4)	—	0.71 (10.0)
18:0/8:0	0.79 (4.8)	—	0.28 (4.0)
16:0/9:0	9.83 (59.5)	—	3.22 (45.5)
18:0/9:0	1.48 (9.0)	—	1.40 (19.8)
MC–PC			
16:0/3:0	0.028 (7.6)	—	—
18:0/3:0	0.0094 (2.8)	—	—
16:0/4:0	0.0067 (1.8)	—	—
18:0/4:0	0.011 (3.3)	—	0.038 (6.9)
16:0/7:0	0.018 (5.4)	—	0.088 (16.1)
18:0/7:0	0.012 (3.6)	—	0.097 (17.7)
16:0/8:0	0.227 (67.8)	0.204 (71.8)	0.235 (42.9)
18:0/8:0	0.057 (17.0)	0.080 (28.2)	0.089 (32.4)

[a]Values in parentheses show percentages of total amount of short-chain dicarboxylate–phosphatidylcholine (DC–PC) or short-chain monocarboxylate (MC)–PC; LDL, low density lipoprotein; DFP, diisopropyl fluorophosphate; PAF, platelet-activating factor.

$n = 3$), 18:0 (13.9 ± 0.5), 18:1 (12.1 ± 1.0), 18:2n-6 (23.7 ± 2.0), 20:3n-6 (3.9 ± 1.0), 20:4n-6 (6.9 ± 1.5), 22:6n-3 (4.0 ± 1.0), and unknown (1.3 ± 2.2). The oxidized LDL contained small amounts of MC–PC with an *sn*-2-octanoyl group, but not those having an *sn*-2-acyl group with a shorter chain than C_7 (Table 1) . This molecular species composition was different from that of MC–PC in oxidized egg yolk LDL. When the plasma LDL was treated with 1 mM DFP and then incubated with Cu^{2+}, it became rich in DC–PC rather than MC–PC, like oxidized egg yolk LDL (Table 1). The MC–PC consisted of species with an *sn*-2-propionyl, and butyryl and heptanoyl groups as well as those with an *sn*-2-octanoyl group, although the amount of the latter was the highest. The composition of molecular species of DC–PC in DFP-treated LDL from human plasma was similar to that in oxidized egg yolk LDL which contained PC having the following fatty acid composition: 16:0 (36.6 ± 1.3 mole%, mean ± SD, $n = 3$), 18:0 (10.4 ± 0.9), 18:1 (26.2 ± 0.6), 18:2n-6 (15.1 ± 1.7), 20:3n-6 (0.9 ± 0.9), 20:4n-6 (2.5 ± 0.7), and 22:6n-3 (6.0 ± 2.2).

PAF-like lipids are unique in terms of imbalance between two fatty acyl tails: a saturated long chain, and a short chain. PAF-like lipids other than MC–PC have an *sn*-2-short chain acyl residue, an end of which is modified by an oxo-, hydroxyl-, or carboxyl-group. This structural imblance should perturb the arrangement of membrane phospholipids when intercalated into the membrane at high concentration, resulting in defects in cell functions, membrane lysis, and cell death. In this study, we compared the effects of different PAF-like lipids ($10^{-6} - 10^{-4}$ M) in inhibiting [^{3}H]thymidine incorpora-

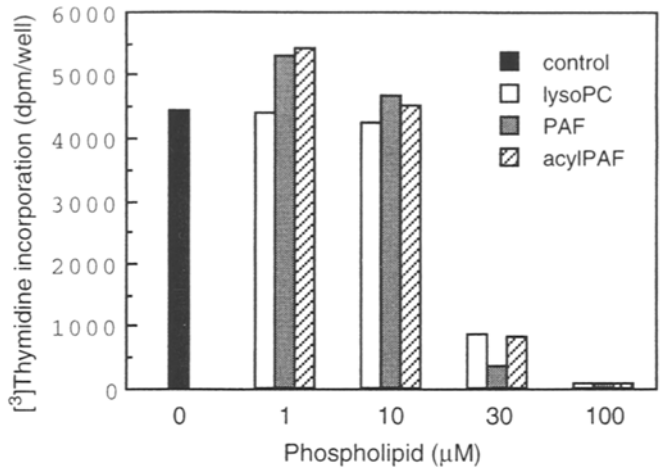


FIG. 2. Effects of different concentrations of platelet-activating factor (PAF), long-chain acyl analogue of PAF (acylPAF), and lysophosphatidylcholine (lysoPC) on [^{3}H]thymidine incorporation into DNA by vascular smooth muscle cells. Results are typical of three separate experiments performed in triplicate, and shown as mean values. Standard errors were between 4–15% of the mean values.

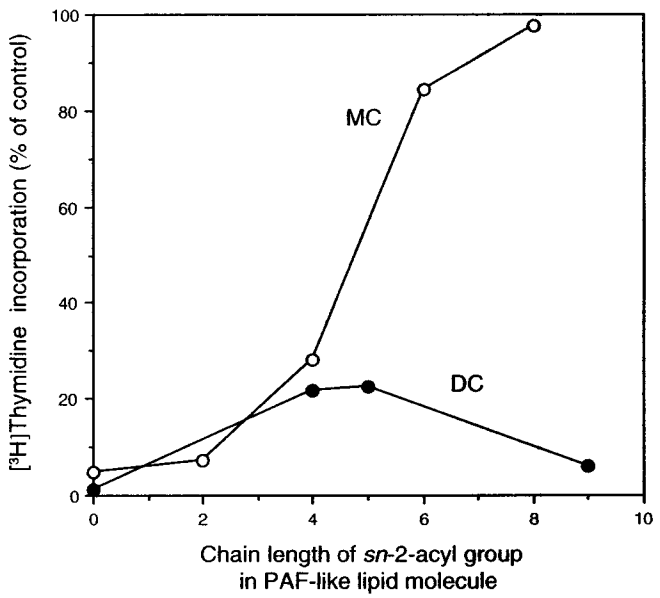


FIG. 3. Structure–activity relationships for inhibitory effects of different short-chain dicarboxylate–phosphatidylcholine (DC–PC) and short-chain monocarboxylate (MC)–PC on [^{3}H]thymidine incorporation into DNA by vascular smooth muscle cells stimulated with 10 ng/mL of platelet-derived growth factor. Results are typical of three separate experiments performed in triplicate, and mean values are expressed as percentages of the control. Standard errors were between 3–12% of the mean values. The concentrations of PAF-like lipids were all 30 μM. See Figure 2 for other abbreviations.

tion into DNA in cultured VSMC stimulated by platelet-derived growth factor. As shown in Figure 2, PAF ($C_{16:0}$) was found to be roughly equipotent to acyl-PAF ($C_{16:0}$) in inhibiting DNA synthesis in VSMC, indicating that the difference between the modes of linkage of the *sn*-1-long chain acyl group to the glycerol backbone was not a critical structural factor for the observed cytotoxic effect, unlike other biological activities expressed *via* PAF receptors such as platelet aggregation (9) and promotion of neutrophil adhesion to a gelatin matrix (27). LysoPC ($C_{16:0}$) also inhibited concentration-dependently the incorporation of thymidine into DNA stimulated by platelet-derived growth factor (Fig. 2). Next, we compared the effects of chain length (*sn*-2) in MC–PC and DC–PC (1, 3, 10, 30, and 100 μM) on their inhibitory actions, and typical results with these phospholipids at 30 μM are shown in Figure 3. With a series of MC–PC, activity decreased with increase in chain length. But, with DC–PC, the effect of an azelaoyl group (C_9) was slightly stronger than those of a succinyl (C_4) or a glutaroyl (C_5) group. These results suggest that the existence of an oxygenated functional group attached to the end of the *sn*-2-acyl group is a more important structural factor than the chain length. FR900452, a PAF receptor antagonist failed to inhibit the cytotoxic effects of DC–PC and MC–PC (data not shown). No significant potentiation by PAF, acylPAF, lysoPC, MC–PC, and DC–PC of platelet-derived growth factor-stimulated thymidine incorporation was observed at any concentrations examined.

DISCUSSION

DC–PC and MC–PC in the Cu^{2+}-oxidized egg yolk LDL were probably generated by homologous β-scission of C–C bonds of intermediate compounds having a reactive alkoxy radical that had been generated from a PC hydroperoxide (28). We suppose that DC(C_9)–PC and MC(C_8)–PC, the most abundant species in each class of PAF-like lipid generated during peroxidation of LDL from egg yolk, are formed by oxidative degradation of 1-palmitoyl-2-linoleoyl-GPC, on the basis of our previous works with synthetic PC peroxidized in Fe^{2+}/EDTA/ ascorbate system (8,9). Similarly, sets of other species of DC–PC (C_8, C_5, C_4) and MC–PC (C_7, C_4, C_3) would be derived from PC having an eicosatrienoate, arachidonate, and docosahexaenoate group, respectively. In fact, the main polyunsaturated fatty acid in LDL from the egg yolk used in this study was linoleate (61.6 mole% of the total polyunsaturated fatty acyl group in the PC), followed by docosahexaenoate (24.5%), arachidonate (10.2%), and eicosatrienoate (3.7%), whereas the percentages of total DC–PC were 68.5, 5.5, 10.8, and 15.2% for the C_9, C_4, C_5, and C_8-species. The reason why the percentages of these DC–PC were not proportional to those of potential parent PC is unclear at present.

When LDL from human plasma was incubated with Cu^{2+} under the same conditions as those for egg yolk LDL, only MC(C_8)–PC was detected in a very small amount, and a considerable amount of lysoPC was found to be accumulated, consistent with earlier reports (25,26). PAF–AH in human plasma has been shown to be bound mainly to LDL (29), and partially-purified PAF–AH has been reported to hydrolyze some PAF-like lipid having a butyryl, glutaroyl, succinyl, or oxovareloyl group (23,30). If PAF-like phospholipids are produced during peroxidation of PC in human plasma LDL, they would be degraded further to lysoPC by the high PAF–AH activity in plasma LDL (23). This possibility was supported by the following findings in the present investigation: (i) Results with [^{3}H]PAF showed reduced PAF–AH activity in DFP-treated LDL. (ii) Peroxidation by Cu^{2+} of human plasma LDL treated with DFP resulted in both the accumulation of DC–PC and MC–PC in comparable amounts to those detected in egg yolk LDL, and the lower level of lysoPC than that in the LDL oxidized in the absence of DFP. DC–PC produced in human plasma LDL seems to be a good substrate for PAF–AH, even when the chain length of the *sn*-2-acyl group is as long as 9. Consistent with previous results on the substrate specificity of PAF–AH in human plasma obtaind using synthetic substrates (23,30), PAF–AH hydrolyzed MC–PC having an acyl group with a chain length of less than 8, because MC(C_8)–PC was detected in similar amounts in oxidized LDL incubated with Cu^{2+} in the presence and absence of DFP.

In this study, we could detect only occasionally PC having a short-chain dicarboxylate semialdehyde residue as a methyloxime derivative (Tokumura, A., *et al.*, unpublished observation), whereas Kamido *et al.* (15) isolated some aldehydic PC as 2,4-dinitrophenylhydrazones from Cu^{2+}-catalyzed peroxidation of human plasma LDL. This inconsistency may be due to the difference in the derivatization procedure, since that the incubation of oxidized LDL with 2,4-dinitrophenylhydrazine could displace protein-bound aldehydes from their Schiff base complexes and convert them, together with the free aldehyde, into the dinitrophenylhydrazones (15), but that the derivatization of PC having an aldehyde moiety to methyoxime was done after its extraction by organic solvent from oxidized LDL. Interestingly, in the presence of phenylmethylsulfonylfluoride, relatively more of the PC having C_5 aldehyde group was shown to be recovered from oxidized human plasma LDL than in the absence of the PAF–AH inhibitor (15), in analogy with our results on MC–PC from the LDL preincubated with and without DFP.

In an advanced stage of atherosclerosis, monocyte-derived macrophages and VSMC migrating to the subendothelium tend to become foam cells by two mechanisms involving oxidized LDL (31). One is excess uptake of oxidatively modified LDL by these cells through a scavenger receptor. The other is the deleterious effect of oxidized LDL on these cells. LysoPC, a possible active component in oxidized LDL, was shown to potentiate [^{3}H]thymidine incorporation into VSMC from rat aorta (32,33) in a narrow range of concentrations, but at higher concentrations to inhibit DNA synthesis (21,33), as Cu^{2+}-oxidized human plasma LDL did (34,35). Thus, in this study, we examined the effects of PAF-like lipids on DNA synthesis in the VSMC, and compared them with that of lysoPC. As expected, the cytotoxic effects of MC–PC with a longer acyl

chain was weaker. In contrast, the cytotoxic effect of DC–PC was independent of the chain length of the *sn*-2-dicarboxylate group. The structure–activity relationship was similar to the apparent substrate specificity of human plasma PAF–AH against oxidatively fragmented PC formed in human plasma LDL. During the preparation of this paper, Heery *et al.* (14) reported that Cu^{2+}-induced oxidation of human plasma LDL resulted in the generation of unknown polar phospholipids that stimulate incorporation of [^{3}H]thymidine into DNA in cultured VSMC from bovine coronary artery and rat aorta, and that these mitogenic effects were inhibited by pretreatment with either a PAF receptor antagonist or PAF–AH. The discrepancy between this interesting report and our study might be due to that platelet-derived growth factor was included in our assay system, but not in that used by Heery *et al.* (14). In this study, our interest was limited to the structure-activity relationships of cytotoxic effects of DC–PC and MC–PC at relatively high concentrations, which were independent of the activation of PAF receptor.

In summary, we demonstrated that PAF-like lipids such as DC–PC and MC–PC were actually generated by peroxidation of human plasma and egg yolk LDL with Cu^{2+}, and confirmed the idea that PAF–AH contributed to the degradation of oxidatively fragmented phospholipids such as PAF-like lipids as well as the inactivation of PAF (23,24). The high ability of PAF–AH to bind to LDL from human plasma, but not to that from egg yolk, favors its clearance preventing the harmful effect of oxidized phospholipids formed in the lipoprotein.

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Oxidation Products of Cyanidin 3-*O*-β-D-glucoside with a Free Radical Initiator

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ABSTRACT: Recently, we have reported that anthocyanins show strong antioxidative activity, but no attention has been paid to anthocyanins from the viewpoint of the reaction mechanism of alkylperoxyl radicals; therefore, we investigated the reaction products of antioxidative anthocyanins (cyanidin 3-*O*-β-D-glucoside). Cyanidin 3-*O*-β-D-glucoside was reacted with 2,2′-azobis(2,4-dimet hylvaleronitrile) to generate the alkylperoxyl radicals, and the reaction products were isolated by high-performance liquid chromatography. The products were identified as 4,6-dihydroxy-2-*O*-β-D-glucosyl-3-oxo-2,3-dihydrobenzofuran and protocatechuic acid. Based on reaction products, the antioxidative mechanism of cyanidin 3-*O*-β-D-glucoside may be different from that of α-tocopherol; cyanidin 3-*O*-β-D-glucoside would produce another radical scavenger, as it would break down the structure and scavenge the radicals.
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Excess production of active oxygen species, such as hydroxyl radicals, superoxide anion radicals, and singlet oxygen and other radicals, is thought to cause damage in cells. This damage is thought to be strongly associated with carcinogenesis, mutagenesis, aging, and atherosclerosis (1–5). Endogenous antioxidants are expected to protect the biological functions of cells (1,6–9). There is increasing interest in the protective biochemical function of natural antioxidants contained in dietary plants, which are candidates for the prevention of oxidative damage caused by oxygen-free radical species (9–12).

Because no attention had been directed to edible beans as natural sources, we previously reported screening for the antioxidative activity of edible beans (13). Among the beans tested, colored pea beans (*Phaseolus vulgaris* L.) had marked activity. These results indicated that pigments contained in the seed coat might play an important role as antioxidants. This background prompted us to investigate the pigments as antioxidants chemically. Three anthocyanin pigments, pelargonidin 3-*O*-β-D-glucoside (P3G), cyanidin 3-*O*-β-D-glucoside (C3G) and delphinidin 3-*O*-β-D-glucoside (D3G), were isolated and identified as antioxidants from red and black beans (*Phaseolus vulgaris* L.) (14–16). Among the pigments isolated, C3G had marked antioxidative activity in a linoleic acid autoxidation system at pH 7.0 (15).

Anthocyanin pigments are widely distributed in the human diet through crops, beans, fruits, and vegetables (17), suggesting that we ingest considerable amounts of anthocyanin pigments from plant-based daily diets. The pigments may play an important role as dietary antioxidants for prevention of oxidative damage caused by active oxygen radicals in living systems. Furthermore, they may be safe and can be used as drugs for some oxidative-damage-induced diseases.

Tocopherols are important antioxidants in food and biological systems, and the reaction products of tocopherols with alkylperoxyl radicals generated from azo-compounds have been isolated and identified. The antioxidative mechanism has been assumed (18–20); however, no studies have ever tried to clarify the antioxidative mechanism of anthocyanins and the reaction products with alkylperoxyl radicals.

In general, anthocyanin pigments are stable under acidic conditions, but they are unstable under neutral or alkaline conditions (21). Interestingly, C3G shows strong antioxidative activity, though at neutral pH, suggesting that flavylium cation form which is concerned with color, may not be essential for expression of the antioxidative activity of C3G.

In the present study, C3G was reacted with an alkylperoxyl radical, which was generated by the reaction of 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) and molecular oxygen, and oxidative products of C3G with AMVN were isolated and identified to clarify the antioxidative mechanism of C3G.

MATERIALS AND METHODS

Chemicals. AMVN and protocatechuic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The commercial food color material, San Red No. 5A, derived from violet corn (*Zea mays* L.) containing C3G, was obtained from San-Ei Gen F.F.I., Inc. (Osaka, Japan). All solvents were distilled in all-glass still before use.

Purification of C3G from commercial food color material. Commercial natural food color material, San Red No. 5A

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Abbreviations: AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); C3G, cyanidin 3-*O*-β-D-glucoside; D3G, delphinidin 3-*O*-β-D-glucoside EI–MS, electron ionization–mass spectra; FAB–MS, fast atom bombardment–mass spectra; HPLC, high-performance liquid chromatography; IR, Infrared; NMR, nuclear magnetic resonance; P3G pelargonidin 3-*O*-β-D-glucoside; TFA, trifluoroacetic acid; UV, ultraviolet; vis, visible.

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(160 mL), was concentrated *in vacuo*, and the crude pigment obtained was used for fractionation in an Amberlite XAD-7 column (45 mm × 400 mm; Organo Co., Ltd., Tokyo, Japan). First, the column was eluted with 1.5 L of distilled water containing 0.1% trifluoroacetic acid (TFA) to remove citric acid and other unwanted substances, and then 1.5 L of methanol containing 0.1% TFA was eluted to obtain the pigment fraction. The eluent was concentrated *in vacuo*, and C3G was isolated from the methanol fraction (5.0 g) using preparative high-performance liquid chromatography (HPLC) with a Develosil ODS-10 column (20 mm × 250 mm; Nomura Chemical Co., Ltd., Aichi, Japan) monitored by a spectrophotometric detector (LC-10AV 530 nm; Shimadzu Works, Co., Ltd., Kyoto, Japan). Acetonitrile (20%) in water containing 0.1% TFA was used as an eluent at a flow rate of 5.0 mL/min. 230 mg of pure C3G (purity, more than 98%) was finally obtained. The structure and purity were confirmed by proton (^{1}H), and carbon (^{13}C) nuclear magnetic resonance (NMR), fast atom bombardment–mass spectra (FAB–MS), ultraviolet (UV)-visible (vis) and infrared (IR) spectra before use.

Reaction procedure. C3G was reacted with AMVN in acetonitrile/distilled water (8:2, vol, vol) at 37°C under air. The final concentrations of C3G and AMVN were 1 and 10 mM, respectively. The reaction mixture was analyzed by HPLC at intervals of 8 h to examine the time course of the reaction. HPLC analysis was carried out using a Develosil ODS-5 column (4.6 mm × 250 mm; Nomura Chemical Co., Ltd., Aichi, Japan) developed with a 30-min linear gradient of water containing 0.1% TFA to acetonitrile containing 0.1% TFA at a flow rate of 1.0 mL/min, and the peak area was calculated by monitoring UV (280 nm). The experiment was run in triplicate and expressed as the average of the area per μL of the reaction mixture.

Color stability of C3G With or Without AMVN. C3G (1mM) with 10 mM or without AMVN in acetonitrile/water (8:2, vol, vol) was incubated at 37°C under air, and the vis absorption (λmax at 530 nm) was measured at intervals of 8 h using a JASCO U-best 50 spectrophotometer (JASCO, Tokyo, Japan). The experiment was run in triplicate, and the result was expressed as the average of the measurements obtained from three independent experiments.

Isolation of reaction products. C3G (225 mg, 0.40 mmol) and AMVN (992 mg, 4.0 mmol) were dissolved in 400 mL of acetonitrile/distilled water (8:2, vol/vol), and the mixture was incubated at 37°C for 24 h in air. After that, the reaction mixture was concentrated *in vacuo*, the residue was dissolved in distilled water, and the crystals of AMVN were removed by filtration. The residue was washed in distilled water three times, and the filtrate was concentrated *in vacuo*; then preparative HPLC was carried out to isolate the reaction products using a Develosil ODS-UG-5 column (10 mm × 250 mm; Nomura Chemical Co. Ltd.) with a UV spectrophotometric detector (LC-10AV, 280 nm; Shimadzu Works, Co. Ltd., Kyoto, Japan). The products were eluted with a 20 min linear gradient ranging from 7% acetonitrile in water containing 0.1% TFA to 60% acetonitrile in water containing 0.1% TFA at a flow rate of 3.0 mL/min.

Instrumental analysis of reaction products. UV absorption spectra were recorded on a spectrophotometer with a JASCO U best-50. IR spectra was recorded on a JASCO FT/IR-8000 with KBr. FAB–MS was recorded on a JEOL JMS-DX-705L (JEOL, Tokyo, Japan) with glycerol or *p*-nitrobenzyl alcohol as the mounting matrix. Electron ionization (EI)–MS was recorded on a JEOL DX-300. ^{1}H NMR and ^{13}C NMR spectra were obtained by a Bruker ARX-400 NMR instrument (400 MHz for ^{1}H and 100 MHz for ^{13}C; Bruker Analytische Messtechnik GmbH, Rheinstetten-Forchheim, Germany) in *d*-CD_3OD containing tetramethylsilane as an internal standard for all isolated products.

RESULTS AND DISCUSSION

Color stability of C3G with or without AMVN. The color stability of C3G with or without AMVN is shown in Figure 1. C3G has the maximum absorption at 530 nm if the flavylium cation form is maintained. When AMVN was not added to the reaction mixture, the absorbance at 530 nm decreased slowly; however, 60% of C3G in the flavylium cation form was still maintained even after 48 h. On the other hand, in the presence of AMVN, the absorbance rapidly decreased, and the color was almost disappeared after 16 h. These results suggest that C3G would react with AMVN; then the flavylium cation structure would be broken by rapid scavenging of the radicals.

Time course for formation of the reaction products. Figure 2 shows the HPLC chromatogram of the reaction products derived from C3G with AMVN in 24 h. Six peaks (P1, P2, P3, P4, P5, and P6) corresponding to the reaction products of C3G appeared on the chromatograms. When C3G was incubated without AMVN in the same condition, some minor peaks were detected at 280 nm by HPLC (data not shown).

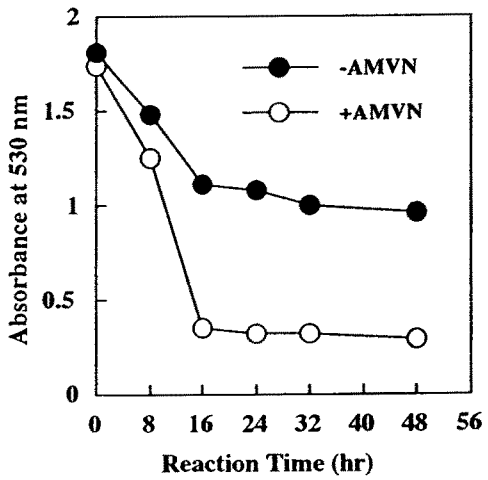


FIG. 1. Absorbance change in cyanidin 3-*O*-β-D-glucoside (C3G) at 530 nm with or without 2,2′-azobis(2,4-dimethylvaleronitrile)(AMVN). The experiment was done in triplicate, and the result was expressed as the average of three independent experiments.

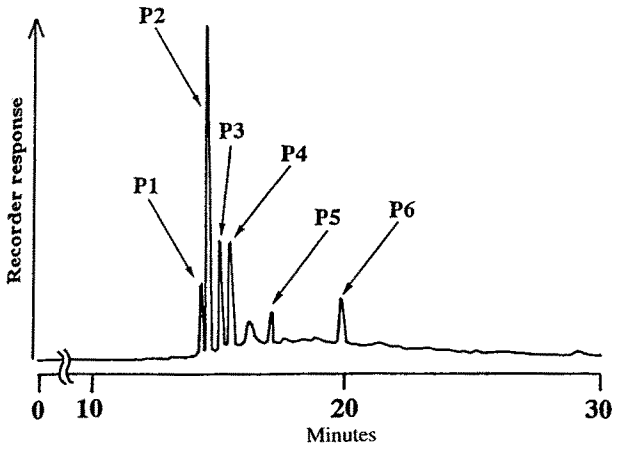


FIG. 2. High-performance liquid chromatography chromatogram of the reaction products derived from cyanidin 3-*O*-β-D-glucoside (C_3G) with 2,2′-azobis(2,4-dimethylvaleronitrile) in 24 h. Conditions: column, Develosil ODS-5 (4.6 mm × 250 mm; Nomura Chemical Co. Ltd., Aichi, Japan); eluent, linear gradient ranging from water containing 0.1% trifluoroacetic acid (TFA) to acetonitrile containing 0.1% TFA over 30 min; flow rate, 1.0 mL/min; detection, ultraviolet (280 nm); P, peak.

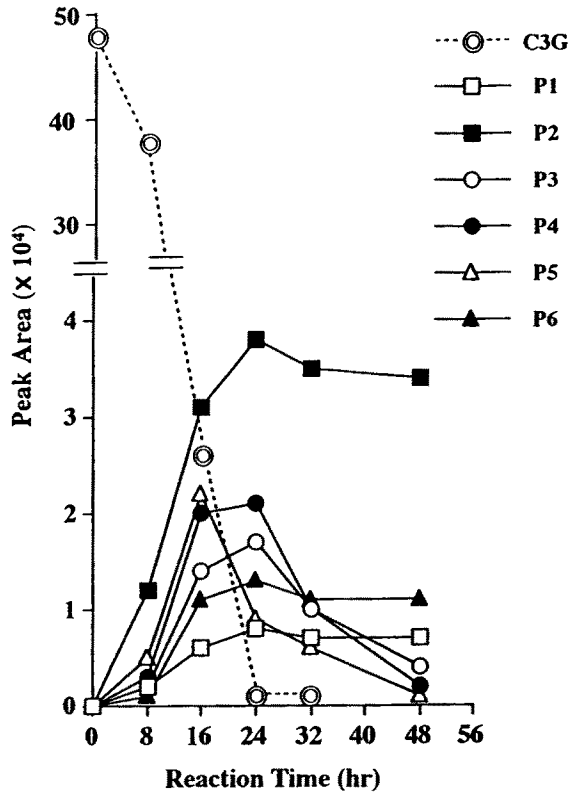


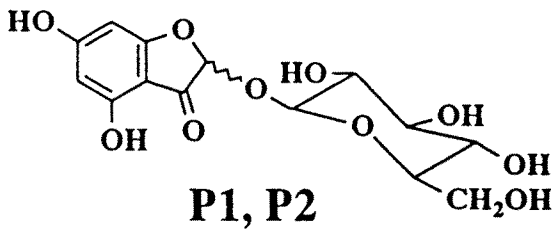
FIG. 3. Time course for the peak areas of the products by the reaction of cyanidin 3-*O*-β-D-glucoside (C3G) with 2,2′-azobis(2,4-dimethylvaleronitrile).

But these peaks were quite different from that of the reaction products with AMVN. Therefore, these products are not due to the autoxidation of C3G. Figure 3 shows the time course for the peak areas of the products. After 16 to 24 h, the formation of the products reached the maximum value, and they decreased beyond this time. Therefore, the reaction was stopped at 24 h, and the reaction products were isolated and identified.

Isolation and identification of the reaction products. The reaction products were purified by preparative HPLC technique, and P1 (4.6 mg), P2 (11.4 mg), P3 (5.8 mg), and P4 (12.7 mg) were isolated.

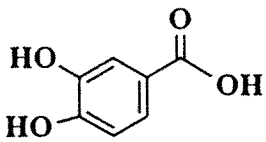
Identification of P1 and P2 (4,6-dihydroxy-2-O-β-D-glucosyl-3-oxo-2,3-dihydrobenzofuran). P1 exhibited the following properties: UV λ_{max} in methanol (nm) 291.5 (log ε = 3.94); IR υ_{max} in KBr (cm^{-1}) 3395.1, 1680.2, 1632.0, 1201.8, 1070.6, 993.5, 835.3; FAB–MS (*m/z*) 345 $[M + 1]^+$, 367 $[M + Na]^+$; ^1H NMR (in *d*-methanol) δ 5.95 (1H, *d*, *J* = 1.6, H_7), 5.91 (1H, *d*, *J* = 1.5, H_5), 5.61 (1H, *s*, H_2), 4.68 (1H, *d*, *J* = 7.6, glucosyl-1), 3.80 (1H, *d*, *J* = 12.0, glucosyl-6), 3.63 (*dd*, *J* = 5.0, 12.0, glucosyl-6), 3.38 (*m*, glucosyl-3), 3.37 (*m*, glucosyl-4, glucosyl-5), 3.28 (*m*, glucosyl-2); ^{13}C NMR (in *d*-methanol) δ 193.3 (*s*, C_3), 174.6 (*s*, C_8), 171.6 (*s*, C_6), 160.3 (*s*, C_4), 103.9 (*d*, glucosyl-1), 102.4 (*d*, C_2), 102.2 (*s*, C_9), 97.8 (*d*, C_5), 91.7 (*d*, C_7), 78.5 (*d*, glucosyl-5), 77.7 (*d*, glucosyl-3), 75.0 (*d*, glucosyl-2), 71.0 (*d*, glucosyl-4), 62.3 (*t*, glucosyl-6). P2 exhibited the following properties: UV λ_{max} in methanol (nm) 290.5 (log ε = 4.13); IR υ_{max} in KBr (cm^{-1}) 3364.3, 1701.4, 1632.0, 1194.1, 1070.6, 993.5, 829.5; FAB–MS (*m/z*) 345 $[M + 1]^+$, 367 $[M + Na]^+$; ^1H NMR (in *d*-methanol) δ 5.93 (^1H, *d*, *J* = 1.8, H_7), 5.90 (1H, *d*, *J* = 1.7, H_5), 5.77 (1H, *s*, H_2), 4.73 (1H, *d*, *J* = 7.8, glucosyl-1), 3.90 (1H, *dd*, *J* = 1.7, 12.0, glucosyl-6), 3.72 (*dd*, *J* = 5.4, 12.1, glucosyl-6), 3.37 (*m*, glucosyl-3), 3.36 (*m*, glucosyl-4, glucosyl-5), 3.28 (*m*, glucosyl-2); ^{13}C NMR (in *d*-methanol) δ 192.7 (*s*, C_3), 175.0 (*s*, C_8), 171.4 (*s*, C_6), 160.3 (*s*, C_4), 102.5 (*s*, C_9), 101.9 (*d*, glucosyl-1), 99.5 (*d*, C_2), 97.6 (*d*, C_5), 91.9 (*d*, C_7), 78.5 (*d*, glucosyl-5), 77.8 (*d*, glucosyl-3), 74.7 (*d*, glucosyl-2), 71.3 (*d*, glucosyl-4), 62.6 (*t*, glucosyl-6). Given these results and considering the two-dimensional NMR spectra, ^1H-^1H correlation spectroscopy, the ^1H-detected multiple quantum coherence spectrum, and the ^1H-detected multiple-bond heteronuclear multiple quantum coherence spectrum (data not shown), P1 and P2 were the diastereoisomers of each other and were identified as 4,6-dihydroxy-2-*O*-β-D-glucosyl-3-oxo-2,3-dihydrobenzofuran.

Identification of P4 (protocatechuic acid). P4 exhibited the following properties: UV λ_{max} in methanol (nm) 294.0 (log ε = 3.59), 259.0 (log ε = 3.84); IR υ_{max} in KBr (cm^{-1}) 3348.8, 1674.4, 1603.0, 1294.4, 1253.9, 943.3, 765.8; EI–MS (*m/z*) 154 $[M]^+$; ^1H NMR (in *d*-methanol) δ 7.44 (1H, *d*, *J* = 2.0, H_2), 7.42 (1H, *dd*, *J* = 2.0, 7.9, H_6), 6.80 (1H, *d*, *J* = 7.9, H_5); ^{13}C NMR (in *d*-methanol) δ 170.2 (*s*, C=O), 151.5 (*s*, C_4), 146.0 (*s*, C_3), 123.9 (*d*, C_6), 123.1 (*s*, C_1), 117.7 (*d*, C_2), 115.7 (*d*, C_5). From these results and the two-dimensional NMR spectral data, ^1H-^1H correlation spectroscopy, ^1H-detected multiple quantum, and ^1H-detected multiple-bond heteronuclear multiple quantum coherence spectrum (data not shown),



P1, P2

4,6-dihydroxy-2-*O*-β-D-glucosyl-3-oxo-2,3-dihydrobenzofuran



P4

protocatechuic acid

FIG. 4. Chemical structures of the oxidative products derived from cyanidin 3-*O*-β-D-glucoside (C3G; see Figure 2 for abbreviation).

P4 could be identified tentatively as protocatechuic acid. To confirm this, instrumental analysis of authentic protocatechuic acid was performed and compared with the data for P4 (data not shown). These authentic spectra were completely consistent with those of P4. Therefore, P4 was identified as protocatechuic acid. The chemical structures of these reaction products are elucidated in Figure 4.

Antioxidative mechanism of C3G. These results suggest the following mechanism for the oxidation of C3G by AMVN (Fig. 5). Water may be added to C3G, and this reaction would be reversible. C3G was reacted with AMVN, and the structure of the flavylium was broken. Group A may transfer hydrogen atoms to peroxyl radicals, oxidation then occurs, one peroxyl radical can be trapped, and the reaction products (P1 or P2) are formed and stabilized. On the other hand, group B would convert into protocatechuic acid (P4), and the radicals may be scavenged by this compound, and resulting in maintaining the antioxidative activity. Therefore, more than two radicals can be trapped by C3G.

α-Tocopherol is a chain-breaking antioxidant and reacts with peroxyl radicals to stop autoxidation of polyunsaturated fatty acids (18). α-Tocopherol transfers hydrogen atoms to peroxyl radicals, and the resulting tocopheroxyl radical rapidly reacts with the peroxyl radical to form peroxide products. Some of the tocopheroxyl radicals react with each other to form dimers and trimers.

On the other hand, C3G may have a different mechanism. The structure of C3G would be broken by peroxyl radicals,

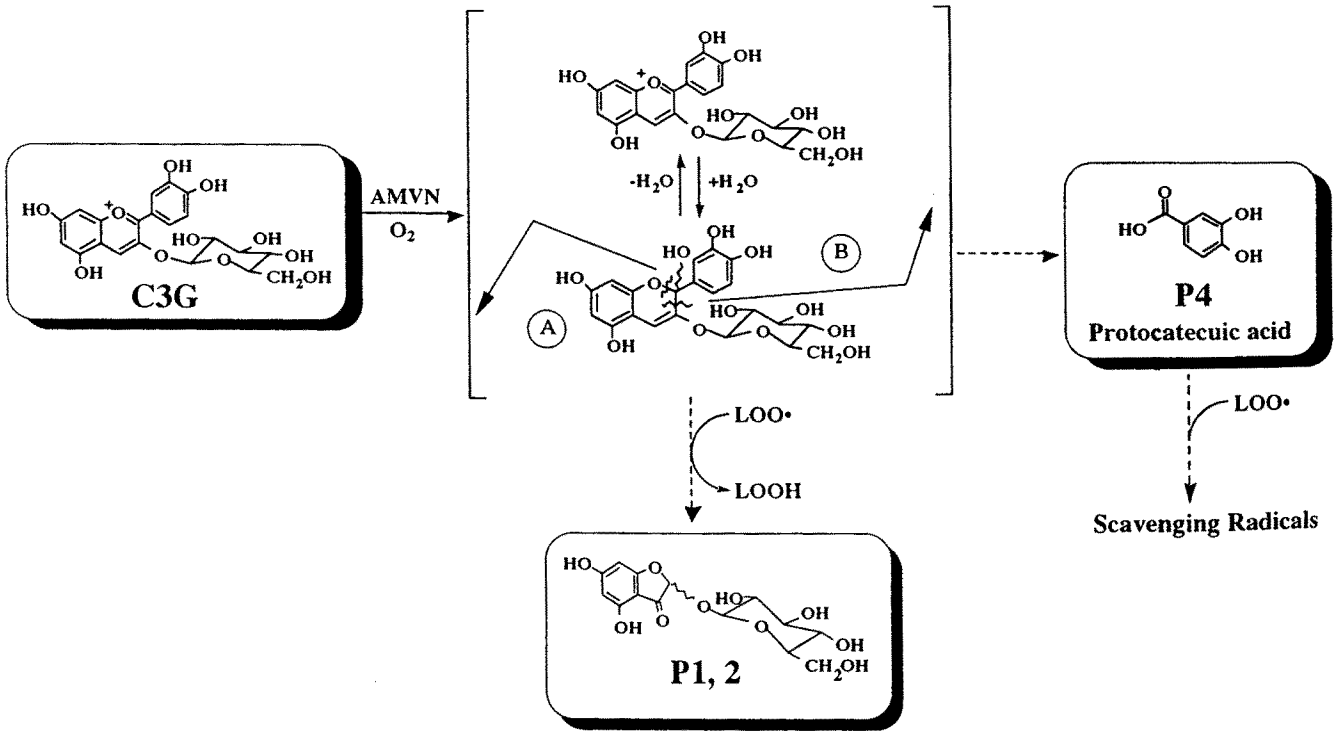


FIG. 5. Proposed mechanism for oxidation of cyanidin 3-*O*-β-D-glucoside (C3G) by AMVN. See Figures 1 and 2 for abbreviations.

the breakdown products may transfer hydrogen atoms to peroxyl radicals, and the products (P1, P2) would be formed and stabilized. During this time, protocatechuic acid, which has antioxidative activity, was also formed from C3G under the reaction conditions. Protocatechuic acid can scavenge the radicals. Namely, C3G would produce another radical scavenger, as it would break down the structure and scavenge the radicals. However, there may be another pathway to scavenge the radicals, because the other product, P3 has not been identified, and scavenging the radicals may be explained on the basis of the mechanism resulting from the reaction pathway through the formation of P3. The identification of P3 is now in progress.

Anthocyanin pigments are widely distributed in the human diet through seeds and crops such as fruits and vegetables. Red wine also has anthocyanins derived from grape skin, indicating that we ingest considerable amounts of anthocyanin pigments from a plant-based daily diet. Anthocyanin pigments are expected to play a role in reducing oxidative damage in cells. However, there is no evidence now that anthocyanins would be effective after food consumption for significantly impacting the antioxidant status of the organism by the antioxidative mechanism we proposed based on the chemical structures of the products. Therefore, it is necessary that the inhibitory effect of anthocyanin pigments against oxidative damage and the mechanism *in vivo* systems be investigated in the future. We plan to carry out a feeding experiment with the anthocyanin pigments using an *in vivo* system.

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Increased Formation of Phosphatidylinositol-4-Phosphate in Human Platelets Stimulated with Lysophosphatidic Acid

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ABSTRACT: Lysophosphatidic acid (LPA, 1-acyl-*sn*-glycerol 3-phosphate), at a concentration of 1–40 μM, was found to induce the formation of [^{3}H]inositol-labelled phosphatidylinositol-4-phosphate (PIP) without significantly altering the levels of either phosphatidylinositol (PI) or phosphatidylinositol bisphosphate (PIP_2) in washed human platelets. Preincubation of platelets with the cyclooxygenase/lipoxygenase inhibitor, BW755C at 100 μM, did not alter the LPA-induced formation of PIP. Activation of platelets with the phorbol ester, phorbol 12-myristate 13-acetate (PMA), elicited a similar response (induction of PIP formation). The specific protein kinase C (PKC) inhibitor, GF109203X (10 μM), completely blocked the effect of PMA but not the LPA-induced generation of PIP. The present results indicate that LPA can induce PIP formation *via* PI-4-kinase activation, through processes which are independent of the eicosanoid/TxA_2 pathway and are not PKC-dependent.
Lipids 31, 1265–1268 (1996).

Lysophosphatidic acid (LPA, 1-acyl-*sn*-glycerol 3-phosphate) is a known agonist for blood platelet aggregation (1–3). LPA is present in human serum (2–20 μM) (4,5) but not in platelet-poor plasma (4), suggestive of its formation during blood clotting. Also, LPA is generated in washed human platelets following stimulation with thrombin (6,7). Studies by Gerrard and Robinson (8) have indicated that, in stimulated human platelets, the action of LPA largely bypasses the phospholipase C-mediated breakdown of phosphoinositides and formation of diacylglycerol and phosphatidic acid.

During the past few years, it has become apparent that platelet exposure to certain agonists including thrombin, collagen, and U46619 induces the formation of phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) *via* the phosphorylation of phosphatidylinositol (PI) and PIP, by the actions of the PI and PIP kinases, respectively (9). These increased phosphoinositide phosphorylations appear to have important and diverse biochemical/cellular functions in mediating human platelet activation (9).

The present studies indicate that LPA markedly enhances PIP formation but not PIP_2 in washed human platelets. Also, this effect of LPA appears to occur independent of thromboxane A_2 (TxA_2)/eicosanoid formation and protein kinase C (PKC).

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Abbreviations: LPA, lysophosphatidic acid; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TLC, thin-layer chromatography; TxA_2, thromboxane A_2.

MATERIALS AND METHODS

Materials. [^{3}H] Inositol (102 Ci/mmol) was purchased from Amersham Canada Ltd. (Oakville, Ontario, Canada). Precoated thin-layer silica gel G chromatographic (TLC) plates were obtained from E. Merck (BDH Chemicals, Toronto, Ontario, Canada). Palmitoyl LPA was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). BW755C was provided by Dr. R. Ippersiel of the Welcome Research Laboratories (Kirkland, Quebec, Canada). GF109203X was obtained from Calbiochem (La Jolla, CA), and phorbol 12-myristate 13-acetate (PMA) was purchased from the Sigma Chemical Co. (St. Louis, MO). All chemicals and solvents were of analytical grade. LPA was dissolved in absolute ethanol, BW755C in distilled water, and PMA and GF109203X were dissolved in dimethyl sulphoxide.

Platelet isolation and prelabelling. Blood was drawn by antecubital venepuncture from healthy volunteers who had not taken any medication for at least 10 d prior to the experiments. Washed platelet suspensions were prepared as outlined (10). Platelet phosphoinositides were prelabelled in the first wash buffer (containing 5 mM HEPES and 4 mM $MnCl_2$) by incubation with 50 μCi of [^{3}H]inositol for two hours at 37°C (10). Platelet counts were adjusted to a final concentration of 5×10^8 platelets/mL in a modified Tyrode's buffer containing 2 mM $CaCl_2$, 1 mM HEPES, and 0.35% essential fatty acid-free bovine serum albumin.

Platelet incubations. One mL aliquots of platelet suspensions were pre-incubated for 1 min in siliconized cuvettes with stirring at 900 rpm at 37°C in an aggregometer (Payton Associates, Buffalo, NY) before activation with 1–40 μM LPA or 40 nM PMA. For the experiments using the inhibitors, the platelet suspension was incubated with BW755C (100 μM) or GF109203X (10 μM) for 1 min, before addition of LPA. All reactions were stopped 2 min after stimulation with LPA or PMA by the rapid addition of 3.75 mL of chloroform/methanol/HCl (20:40:1, by vol), and the phospholipids were extracted.

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Extraction and analysis of phosphoinositides. Phospholipids including PIP and PIP_2 were extracted according to the method of Allan and Michell (11). Thin-layer separation of [^{3}H]PI, [^{3}H]PIP, and [^{3}H]PIP_2 and scintillation counting of these bands were as described in an earlier paper (10). In isolated studies, further separation of the PI-3-P and PI-4-P isomers was obtained as follows. The PIP band was scraped, eluted, and run twice on an oxalate-impregnated TLC plate using the solvent system chloroform/methanol/NH_4OH/H_2O (40:45:9:5, by vol).

Statistical analysis. The data was analyzed using a general linear model. Specific differences between treatments were analyzed by Student's *t*-tests (12).

RESULTS AND DISCUSSION

Figure 1 shows that LPA induced a time- and dose-dependent increase in the levels of [^{3}H]PIP. The effect was optimal when platelets were activated with 20 μM LPA for 2 min. There was no change in the levels of [^{3}H]PI, although [^{3}H]PIP_2 showed a slight (but not significant) decrease for up to 1 min of activation. This may indicate an early activation of phospholipase C, followed by rapid turnover of the substrate by the actions of the PI- and PIP-kinases. The present work indicates that LPA, as a platelet agonist, can induce the formation of PIP without enhancing PIP_2 accumulation, possibly *via* the enhancement of PI-kinase activity. Preincubation of platelets with the cyclooxygenase/lipoxygenase inhibitor, BW755C (100 μM), did not significantly alter the agonist-induced formation of PIP (the corresponding mean values for [^{3}H]PIP upon LPA exposure were 151.8 and 151.7% of resting values in the absence and presence of BW755C, respectively). At this concentration, BW755C has been previously shown to completely block eicosanoid production (13). Thus, this LPA-induced rise in PIP appears to occur *via* eicosanoid/TxA_2-independent mechanisms. Further analysis of the PIP band, *via* TLC separation of PI-4-P from PI-3-P, showed that most of the radioactivity was associated with the PI-4-P fraction, indicating an activation of PI-4-kinase. For subsequent routine experiments, 10 μM LPA and a 2-min incubation time were employed.

Activation of platelets with 1-oleoyl-2-acetyl-glycerol (14) and with PMA (15) have been reported to cause significant increases in the levels of PIP and, to a lesser extent, in PIP_2. Table 1 shows that activation of platelets with the phorbol ester, PMA (40 nM, 2 min), increased the generation of PIP (by 188%), without significantly affecting the levels of PIP_2. This pattern was similar to that obtained with LPA. It has been suggested that one of the roles of PKC would be the induction, by phosphorylation, of PI-kinase activity (15). Therefore, to test if the effects of LPA were mediated by PKC, platelets were preincubated with the specific PKC inhibitor GF109203X (10 μM) before activation with either LPA or PMA (16). It was observed that at this concentration GF109203X completely blocked the aggregation of platelets induced by both PMA as well as LPA (data not shown). However, it was interesting to note that, whereas GF109203X also blocked the effect of PMA in enhancing PIP formation, it had no effect on the LPA-induced generation of PIP. The latter results suggest that the LPA stimulation of PIP production *via* PI-4-kinase is PKC-independent. Further, it appears that in LPA-stimulated platelets, the formation of PI-4-P and platelet aggregation are not related events.

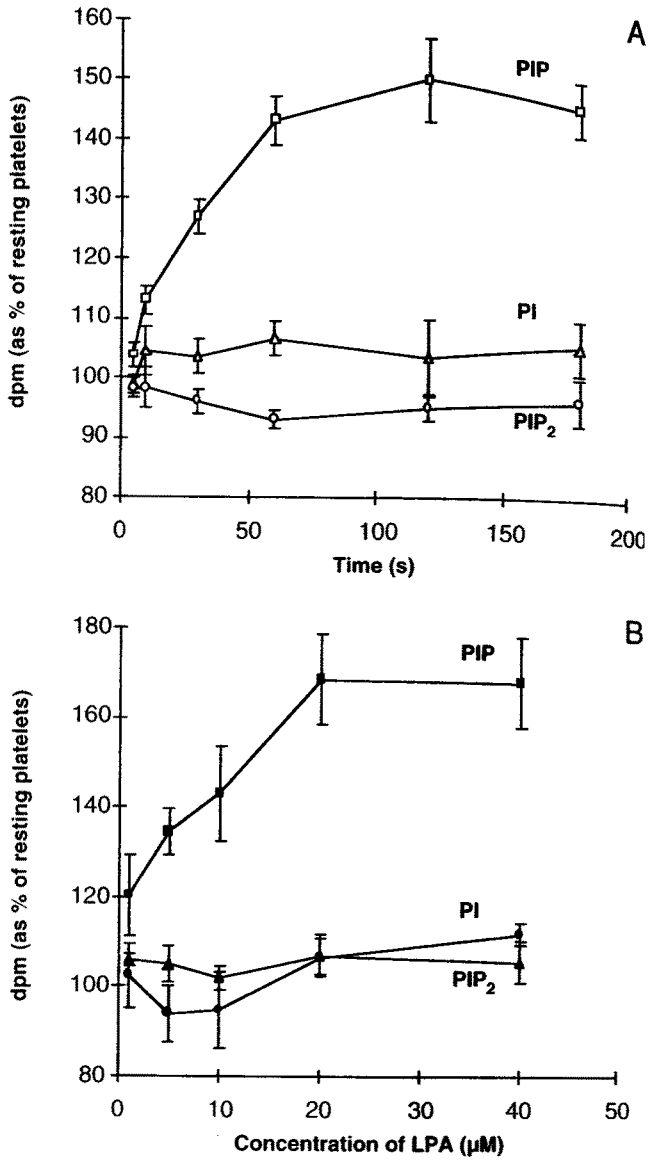


FIG. 1. Washed human platelets prelabelled with [^{3}H]inositol were incubated with (A) 10 μM LPA for different periods of time (5, 10, 30, 60, 120, and 180 s) or (B) different concentrations of LPA (1, 5, 10, 20, and 40 μM) for 1 min, before the reactions were stopped and lipids extracted as described in the Materials and Methods section. The different phosphoinositides were separated by TLC, and the areas corresponding to PI, PIP, and PIP_2 were scraped and counted in a scintillation counter. Values are reported as mean ± SEM (from three experiments performed in duplicate). The resting dpm values for PI, PIP, and PIP_2 were 12720 ± 511, 1776 ± 141, and 1377 ± 106, respectively.

TABLE 1
Effect of the Protein Kinase C Inhibitor GF109203X on PMA- or LPA-Induced Formation of [^{3}H]Inositol-Labelled Phosphoinositides[a]

Phospholipid	Radioactivity in resting platelets (dpm)	Radioactivity in stimulated platelets (as % of resting platelets) PMA (40 nM)	GF (10 μM) + PMA (40 nM)	LPA (10 μM)	GF (10 μM) + LPA (10 μM)
PIP	1892 ± 19	287.6 ± 15.2**	118.6 ± 4.7[b]	143.1 ± 4.1*	146.3 ± 1.0*
PIP_2	2653 ± 95	124.4 ± 5.7	110.1 ± 2.6	96.2 ± 1.5	112.5 ± 1.4

[a]Significantly different **($P < 0.001$) and *($P < 0.01$) from unstimulated values.
[b]Significantly different ($P < 0.001$) from corresponding values in the absence of the inhibitor. Values are reported as mean ± SEM (n = four experiments performed in triplicate). PMA, phorbol 12-myristate 13-acetate; LPA, lysophosphatidic acid; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate.

The finding herein, that LPA activates PI-4-kinase, is of interest since it has been reported that PI-4-kinase is associated with the transformation and proliferation of hepatoma cells. Olson (17) has reported enhanced PI-kinase activity in the early stage of hepatocarcinogenesis and Rizzo and Weber (18) showed that there is a marked increase in the activity of PI-4-kinase in transplantable hepatomas and in sarcoma. Although the results shown here are in platelets, which are not proliferating cells, it is possible that LPA causes the same effect of increasing PI-4-P levels in other cells too. Thus the results of the present study lead us to speculate that the reported mitogenic properties of LPA (19,20) may stem, in part, from its ability to activate the enzyme PI-4-kinase.

At the time of preparation of this manuscript, Zhang and Rittenhouse (21) reported an activation of PI-3,4-P_2 and PI-3,4,5-P_3 formation *via* phosphoinositide 3-kinase in LPA-stimulated human platelets. However, in that study, the authors did not report upon PIP formation. We too have confirmed an LPA-induced rise in PI-3,4-P_2 (data not shown); this phosphoinositide represents approximately 8% of the total radioactivity in the total PIP_2 fraction (the remaining 92% being represented by PI-4,5-P_2).

In conclusion, the present findings indicate that LPA can markedly induce the formation of PI-4-P *via* activation of PI-4-kinase in washed human platelets. This LPA-induced formation of PI-4-P appears to occur independent of TxA_2/eicosanoid formation and PKC activation. However, the specific biochemical mechanisms regulating this formation of PI-4-P remain to be elucidated.

ACKNOWLEDGMENT

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Differential Inhibitory Effects of Garlic-Derived Organosulfur Compounds on Cholesterol Biosynthesis in Primary Rat Hepatocyte Cultures

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ABSTRACT: Using primary rat hepatocyte cultures, the potency of several garlic-derived organosulfur compounds to inhibit cholesterol biosynthesis *in toto* as well as at early and late steps of this metabolic pathway was compared. Concerning early steps, allicin significantly inhibited incorporation of [^{14}C]acetate into nonsaponifiable neutral lipids already at concentrations as low as 10 μM, while diallyl disulfide and allyl mercaptan were effective above 100 μM only. Likewise, inhibition in response to the two vinyl-dithiins started at 500 μM. If [^{14}C]acetate was replaced by [^{14}C]mevalonate, inhibition due to allicin, diallyl disulfide, and allyl mercaptan disappeared suggesting that HMGCoA-reductase was the target of inhibition. In contrast, for the vinyl-dithiins a stimulation of mevalonate incorporation was found. Concerning the late step, the potency to exert accumulation of lanosterol presumably by inhibiting lanosterol 14α-demethylase decreased in the order allicin > diallyl disulfide > allyl mercaptan = 1,3-vinyl-dithiin >> 1,2-vinyl-dithiin, the effect of the latter compound being close to zero. With respect to the total inhibition of [^{14}C]acetate labeling of cholesterol, the half-maximal effective concentration-value of allicin was determined to be 17 ± 2 μM compared to 64 ± 7 μM for diallyl disulfide and to 450 ±20 μM for allyl mercaptan. Cytotoxicity as determined by the lactate dehydrogenase leakage assay was slightly higher for the two vinyl-dithiins than for diallyl disulfide and allyl mercaptan, but was apparent only at concentrations higher than 10 mM and, consequently, was irrelevant for the effects described. These results demonstrate that different garlic-derived organosulfur compounds interfere differently with cholesterol biosynthesis and, thus, may provoke multiple inhibition of this metabolic pathway in response to garlic consumption. The fact that allicin was the most effective inhibitor argues against the possibility that its degradation products, namely diallyl disulfide or allyl mercapatan, might mediate its effects, a possibility that might be true, however, in the case of the vinyl-dithiins.

Lipids 31, 1269–1276 (1996).

Garlic preparations have long been known to exert lipid-lowering effects in animals and humans (1,2). Recently, significant reduction of blood cholesterol after long-term intake of garlic powder tablets has been found for humans in double-blind placebo controlled studies (3–8). Moreover, when dosed appropriately, garlic preparations (Sapec^{R}, Kwai^{R}, Lichtwer Pharma GmbH, Berlin, Germany) were shown in a randomized double-blind study to act as efficient as the hypolipidemic drug bezafibrate in reducing total cholesterol, low density lipoprotein (LDL) cholesterol and increasing high density lipoprotein (HDL) cholesterol in a collective of patients with primary hyperlipoproteinaemia (9). Contrasting with these consistent results, little is known as yet about the mechanism(s) and the active principle(s) responsible for these effects.

Garlic preparations contain a wide variety of organosulfur compounds which are mainly derived from alliin (1,2,10). The spectrum of these sulfur-containing compounds depends considerably upon the method of drying or extraction of the original garlic bulbs, as well as upon the temperature, pH, and solvent conditions of subsequent processing. Thus, incubation in aqueous solutions of allicin, the main thiosulfinate, predominantly results in the formation of diallyl disulfide and diallyl trisulfide (11,12). In low-polarity solvents, on the other hand, mainly 1,3-vinyl-dithiin (2-vinyl-4*H*-1,3-dithiin) and 1,2-vinyl-dithiin (3-vinyl-4*H*-1,2-dithiin) are formed (11,13).

Some of these compounds have been tested in animal studies (14,15) or under *in vitro* conditions (16,17), but because of the high concentrations used it seems hazardous to draw conclusions about their physiological effectiveness. Under artificial, but nearly physiological conditions, further reactions of all these compounds may occur, particularly with cysteine (for review, see Ref. 2), thus complicating the attribution of specific effects to particular compounds. However, since allyl mercaptan is a common final product of most of these reactions, it has been suggested that this compound might play a central role in mediating the effects of garlic-derived thiosulfinates (2).

Recently, rat hepatocyte cultures have proved suitable for investigating the effects of water-soluble garlic extracts (18–21). Using this experimental system, the effects of the garlic-derived thiosulfinates allicin and ajoene on cholesterol biosynthesis could be described in detail (22). There was not

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Abbreviations: AM, allyl mercaptan; DADS, diallyl disulfide; DMSO, dimethyl sulfoxide; EC50, half-maximal effective concentration; HMGCoA-reductase, hydroxymethylglutaryl-CoA-reductase (EC 1.1.1.34); HPLC, high-performance liquid chromatography; LDH, lactate dehydrogenase (EC 1.1.1.27); SI–TLC, silver-ion–thin-layer chromatography; 1,2-VDT, 3-vinyl-4*H*-1,2-dithiin; 1,3-VDT, 2-vinyl-4*H*-1,3-dithiin.

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only a reduction of the incorporation of acetate into nonsaponifiable lipids by ajoene in the range of 18%, but also a shift in the pattern of sterol compounds indicating an inhibition at the level of lanosterol 14α-demethylase by both thiosulfinates (22). In the present study, we have performed comparative experiments on the effects of several other organosulfur compounds including diallyl disulfide, allyl mercaptan, and the vinyl-dithiins with those of allicin. These experiments were designed to provide information whether or not the inhibitory effect of garlic-derived thiosulfinates may be ascribed to the formation of common final metabolic products such as allyl mercaptan. It is reported that each of the compounds tested exerts a different spectrum of interactions with the biosynthetic pathway of cholesterol which only partially overlaps with that produced by allicin.

MATERIALS AND METHODS

Materials. Diallyl disulfide and allyl mercaptan were purchased from Aldrich (Steinheim, Germany). Allicin as well as the vinyl-dithiins, 1,3-vinyl-dithiin (2-vinyl-4*H*-1,3-dithiin) and 1,2-vinyl-dithiin (3-vinyl-4*H*-1,2-dithiin) were a kind gift of Prof. Dr. K.G. Wagner, (Braunschweig, Germany). The structure of these compounds is depicted in Figure 1. The purity of the inhibitors was routinely checked by high-performance liquid chromatography (HPLC). Only materials containing less than 2% impurities were used for the experiments. [U-^{14}C]Acetate, sodium-salt, [1.9 GBq/mmoles (51.3 mCi/mmoles)] and RS-[2-^{14}C]mevalonic acid, dibenzylethylendiamine salt, [2.1 GBq/mmoles (56.7 mCi/mmoles)] were obtained from Amersham/Buchler (Braunschweig, Germany). Collagenase (5,6 U/mg with Azokoll) was from Knoll AG (Ludwigshafen, Germany), and was used in batches which proved superior for the isolation of hepatocytes in our laboratory. Newborn calf serum was obtained from Vitromex (Vilshofen, Germany); Williams Medium E was from ICN Biochemicals (Meckenheim, Germany). The Extrelut^{R}20-columns were from Merck (Darmstadt, Germany). All other chemicals were from Boehringer (Mannheim, Germany), Merck, or Sigma (Daisenhofen, Germany).

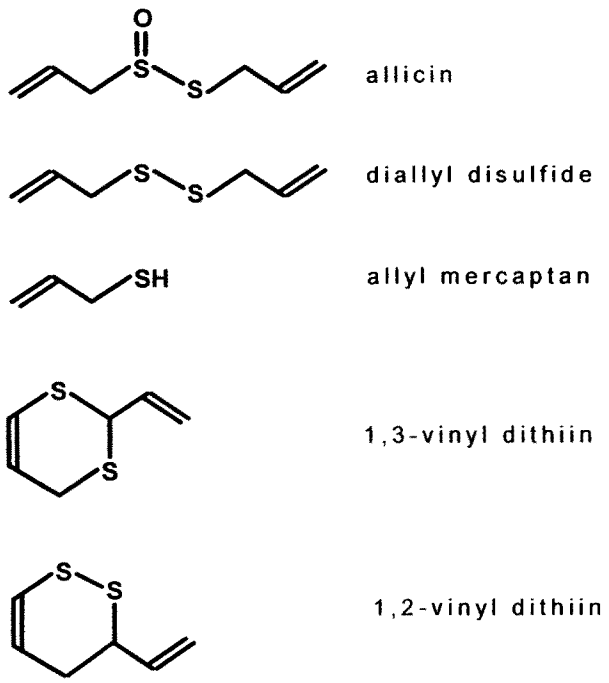


FIG. 1. Structure of garlic-derived organosulfides used in this study.

Animals. Male Sprague-Dawley rats (220–300 g) were used as hepatocyte donors. They were kept in a controlled 12-h light-and-dark cycle on a standardized diet ofAlma^{R} H 1003 (Botzenhardt, Kempten, Germany) and tap water *ad libitum*. Alma^{R} H 1003 is prepared from wheat, oilseeds, milk, and materials from sugar refinery. The macronutrient composition is as follows: 19% raw protein, 4% raw fat, 23% raw carbohydrates, 6% fibers, 7% ash, 1% calcium, 0.8% phosphorus, 0.5% sodium, 0.2% magnesium, 0.4% methionine, and 1.2% lysine. It is supplemented with 15.000 I.E. vitamin A, 500 I.E. vitamin D_3, 150 mg vitamin E, and 100 mg vitamin C per kg.

Isolation and cultivation of rat hepatocytes. Rat hepatocytes were isolated according to the two-step collagenase perfusion technique described (23). Viability of the cells averaging to 91.8 ± 3.1% was routinely checked by staining with trypan blue. The isolated hepatocytes were suspended in Williams E medium containing 10% newborn calf serum, 2 mM glutamine, penicillin (50 Units/mL), streptomycin (50 μg/mL), and 10^{-7}M dexamethasone (18,24). They were seeded in 2 mL of culture medium at a density of $1.25 \cdot 10^5$ cells/cm^2 into Petri dishes (O 60 mm) precoated with a thin film of collagen (25) and were incubated at 37°C, 90% humidity, and 7% CO_2. From 2 h on, serum-free medium was used.

Incubation of the primary cultures with purified garlic-derived organosulfur compounds. Pure diallyl disulfide, allyl mercaptan, and allicin were dissolved or diluted directly in Williams medium E to give concentrations of 1 mM or lower. Higher concentrations had to be prepared by dissolving the compounds in DMSO before Williams medium E could be added. The vinyl-dithiins were also diluted with DMSO before addition of the medium. The final concentrations of DMSO never exceeded 2%, and appropriate controls containing DMSO were run. All media were sterilized by filtration before use. Dilutions were always made with serum-free medium.

Two hours after inoculation of the hepatocyte cultures, the medium was removed and fresh medium was added containing [^{14}C]acetate [18.5 KBq/mL (0.5 μCi/mL)] and the compounds to be tested in the appropriate dilutions. After incubation at 37°C for additional 2 h, the medium was removed and the cell-layer was washed twice with saline and scraped into 2 mL H_2O distilled. The cells were homogenized by sonication (20 s, grade 3, (19,20)]. In several experiments, [^{14}C]acetate was replaced by [^{14}C]mevalonate [9 KBq/mL (0.24 μCi/mL)].

Determination of acetate incorporation into and separation of nonsaponifiable neutral lipids. The incorporation of

[^{14}C]acetate into nonsaponifiable neutral lipids was determined after saponification with 0.5 M KOH in EtOH and subsequent efficient separation on Extrelut^{R}-columns (large-pore kieselgur) as described (18,19). The neutral lipophilic nonsaponifiable substances were eluted with *n*-heptane (18,26). The precursors such as [^{14}C]acetate or [^{14}C]mevalonate are retained on the column to more than 99%. For measurements of incorporation, the eluate was collected directly in scintillation vials and measured in the scintillation counter after addition of 10 mL of Ultima Gold^{R} (Packard, Merident, CT). The yield of the elution step (93 ± 2%) and the recovery were determined as described (19).

Determination of the sterol composition of the nosaponifiable neutral lipid fraction. For determination of the newly synthesized sterol pattern, the eluate was evaporated to dryness in a vacuum concentrator (Desaga, Heidelberg, Germany), taken up in 50 µl chloroform and applied to silver-ion thin-layer chromatography (SI–TLC) plates (silica gel 60F 254; Merck) which were impregnated with silver nitrate and activated as described by Aufenanger *et al.* (26). They were developed only once using a mixture of *n*-heptane/ethyl acetate (2:1, vol/vol) on a total length of 16 cm according to Aufenanger *et al.* (26) and Pill *et al.* (27). The amount of radioactivity was determined by scraping stripes of 2 mm into vials for scintillation counting. This technique provided quite similar results as radiochromatographic scanning of the TLC plates as determined in separate validation experiments. With [^{14}C]mevalonate, a similar protocol was used. Alternatively, the sterol pattern was determined by HPLC on ODS Ultrasphere (0.46 × 25 cm) (Grom, Herrenberg, Germany) in a mobile phase of methanol/ethanol/water (86:10:4) according to Mayer *et al.* (28) with collecting fractions from the column and detection of radioactivity by scintillation counting.

Determination of cytotoxicity and other analytical procedures. Cytotoxicity of the compounds tested was determined by means of the lactate dehydrogenase (LDH) leakage assay as described (21,29). Protein was determined following the procedure of Lowry *et al.* (30).

Statistical evaluation. The EC_{50} values (half-maximal effect) were determined from the dose-response curves by use of curve-fitting programs [Fig. P and PENNZYME (31), (obtained from authors)] on a personal computer. The data were evaluated statistically using Student's *t*-test or analysis of variance. Data are given as means ± standard deviation.

RESULTS

In order to avoid any unspecific effects on the inhibition of cholesterol biosynthesis, we have directly determined the cytotoxic potential of diallyl disulfide, allyl mercaptan, and of the two vinyl-dithiins (structures see Fig. 1) in primary rat hepatocytes using the LDH leakage assay. As shown in Table 1, the two vinyl-dithiins were slightly more cytotoxic than diallyl disulfide or allyl mercaptan for rat hepatocytes. For all these compounds, however, cytotoxicity started at concentrations above 10 mM. Therefore, the exact half-maximal effective concentrations could not be determined because of the limited solubility of the compounds. Allicin, on the other hand, was more cytotoxic starting to affect the hepatocytes at 0.5 mM (22).

TABLE 1
Cytotoxicity of Different Garlic-Derived Organosulfides on Cultured Rat Hepatocytes

Concentration of compounds used (mM)	LDH-leakage (% total activity)[a]			
	DADS	AM	1,2-VDT	1,3-VDT
Control	5 ± 1	6 ± 2	5 ± 2	5 ± 2
0.1	6 ± 2	5 ± 2	5 ± 1	5 ± 3
0.5	5 ± 1	4 ± 3	5 ± 2	6 ± 1
1	6 ± 1	5 ± 1	7 ± 2	4 ± 3
5	7 ± 3	6 ± 2	9 ± 3	6 ± 2
10	5 ± 2	8 ± 3	13 ± 2[c]	8 ± 2[b]
15	8 ± 2[b]	17 ± 4[c]	29 ± 5[c]	16 ± 4[c]
20	12 ± 2[c]	22 ± 3[c]	34 ± 8[c]	27 ± 4[c]

[a]Data represent means ± SD of triplicate determinations of lactate dehydrogenase (LDH) (EC 1.1.1.27) leakage. DADS, diallyl disulfide; AM, allyl mercaptan; 1,2-VDT, 3-vinyl-4*H*-1,2-dithiin; 1,3-VDT, 2-vinyl-4*H*-1,3-dithiin.
[b]Significant cytotoxicity, $P < 0.05$.
[c]Significant cytotoxicity, $P < 0.01$.

Incubation of primary rat hepatocyte cultures with allicin in concentrations between 0.1 and 1000 µM as early as 2 h after cultivation caused significant inhibition of the incorporation of [^{14}C]acetate into nonsaponifiable neutral lipids at concentrations above 10 µM (Fig. 2) suggesting inhibition before the formation of squalene. Diallyl disulfide and allyl mercaptan were less effective and inhibited at concentrations above 100 µM only. At 1 mM, inhibition amounted to approximately 35, 25, and 15% for allicin, diallyl disulfide, and allyl mercaptan, respectively (Fig. 2). When diallyl disulfide and allyl mercaptane were combined, their influence was close to that of twice the amount of each compound (Table 2).

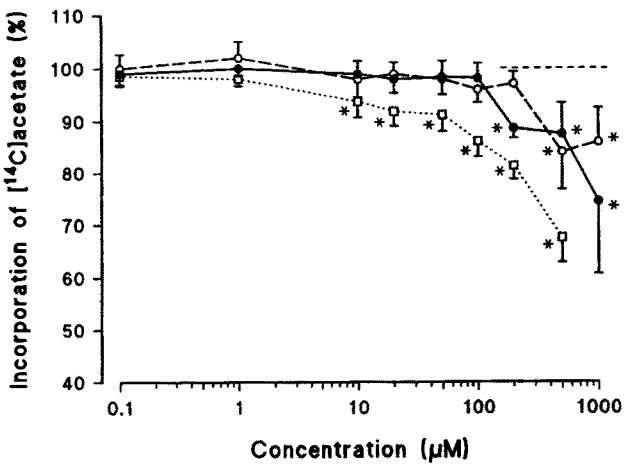


FIG. 2. Dependence of the incorporation of [^{14}C]acetate into nonsaponifiable lipids of cultured rat hepatocytes on the concentration of allicin (□···□), diallyl disulfide (●—●) and allyl mercaptan (○–○). The dashed line on the right indicates the control level. Data represent means ± SD of three to five independent determinations and are expressed as percentage control. *, Statistically different from controls, $P < 0.01$.

TABLE 2
Inhibition of the Biosynthesis of Nonsaponifiable Neutral Lipids from [^{14}C]Acetate or [^{14}C]Mevalonate by Diallyl Disulfide and Allyl Mercaptain in Rat Hepatocytes

Inhibiting material	Concentration (μM)	Inhibition (% of control)[a]	
		[^{14}C]Acetate[b]	[^{14}C]Mevalonate
Allicin	50	11 ± 3	2 ± 1[c]
	500	32 ± 5[d]	4 ± 2[c]
Diallyl disulfide	100	3 ± 3	2 ± 1
	250	9 ± 4[e]	2 ± 1[c]
	500	15 ± 6[e]	3 ± 2[c]
Allyl mercaptan	100	4 ± 2	2 ± 2
	250	8 ± 3[e]	2 ± 2[c]
	500	13 ± 5[e]	2 ± 2[c]
Diallyl disulfide + Allyl mercaptan	250		
	250	15 ± 4[e]	2 ± 2[c]

[a]Values represent means ± SD of tripicate determinations. Inhibition is expressed as percentage of control values.
[b]Incorporation of radioactivity for controls was 12.3 ± 1.4 dpm/μg protein and 7.1 ± 0.7 dpm/μg protein for [^{14}C]acetate and [^{14}C]mevalonate, respectively.
[c]Significantly different from acetate: $P < 0.01$.
[d]Significantly different from control: $P < 0.001$.
[e]Significantly different from control: $P < 0.01$.

There was no synergistic effect throughout the concentration range used.

As for diallyl disulfide, incubation of the hepatocytes with 1,3-vinyl-dithiin or 1,2-vinyl-dithiin also did not significantly influence [^{14}C]acetate incorporation at concentrations below 100 μM (Fig. 3). At 1 mM, a strong inhibitory effect was found which was more pronounced in the case of 1,3-vinyl-dithiin than for 1,2-vinyl-dithiin (Fig. 3).

If [^{14}C]acetate was replaced by [^{14}C]mevalonate, the inhibitory effect of allicin disappeared to a large extent (Table 2), indicating that inhibition occurs even earlier at some enzymatic step prior to the formation of mevalonate. The same was seen with diallyl disulfide and allyl mercaptan (Table 2). In contrast, 1,3-vinyl-dithiin significantly stimulated sterol synthesis from mevalonate between 1 and 10 μM (Fig. 4). 1,2-vinyl-dithiin seemed to be slightly more potent, although its effect at 0.1 μM ($P < 0.06$) was not as significant as at 10 μM ($P < 0.02$) (Fig. 4). Inhibition was either not observed (1,3-vinyl-dithiin) or occurred only at millimolar concentrations (1,2-vinyl-dithiin).

Recently, allicin and ajoene but not alliin were found to inhibit cholesterol biosynthesis not only at an early enzymatic step (HMGCoA-reductase) but also at a later step of the pathway, namely at the level of lanosterol 14α-demethylase (22). Based on structural considerations, we hypothesized that the allylsulfide moiety within these molecules might be of importance for this inhibition (Gebhardt, R., unpublished observation). Since this structural component is found in some of the organosulfur compounds studied herein, we determined whether these compounds might also inhibit this enzymatic step.

Analysis of the sterol pattern produced by the rat hepatocytes by SI–TLC (26) or HPLC consistently revealed that

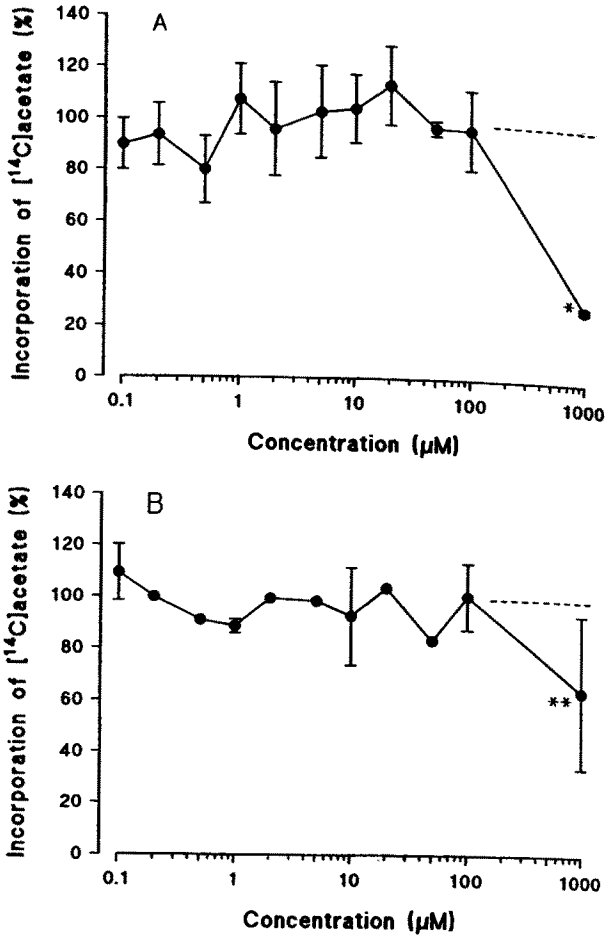


FIG. 3. Dependence of the incorporation of [^{14}C]acetate into nonsaponifiable lipids of cultured rat hepatocytes on the concentration 1,3-vinyl-dithiin (A) and of 1,2-vinyl-dithiin (B). The dashed line on the right indicates the control level. Data are expressed as percentage control and represent means ± SD of three to four independent determinations except the points without SD bars in panel B which were from one experiment only. *, Statistically different from controls, $P < 0.001$. **, Statistically different from controls, $P < 0.05$.

cholesterol was the main sterol formed from [^{14}C]acetate (Figs. 5A,B). The precursors lanosterol and 7-dehydrocholesterol amounted to 8 and 4%, respectively. As obvious from Figure 5, diallyl disulfide at concentrations above 10 μM blocked the formation of 7-dehydrocholesterol, reduced the production of cholesterol, and gave rise to corresponding amounts of lanosterol and, to a lesser extent, dihydrolanosterol. At 1 mM, almost all label derived from [^{14}C]acetate was found in lanosterol. Apart from these three compounds, other precursors contained very little label only, usually less than 8% (Fig. 5A) as confirmed independently by HPLC analysis. The results indicate that diallyl disulfide affected mainly lanosterol 14α-demethylase. Allyl mercaptan produced similar changes of the sterol pattern, but was effective only at concentrations above 200 μM (Fig. 5B).

Direct comparison of the potency of allicin, diallyl disulfide, allyl mercaptan, and the vinyl-dithiins to cause accumu-

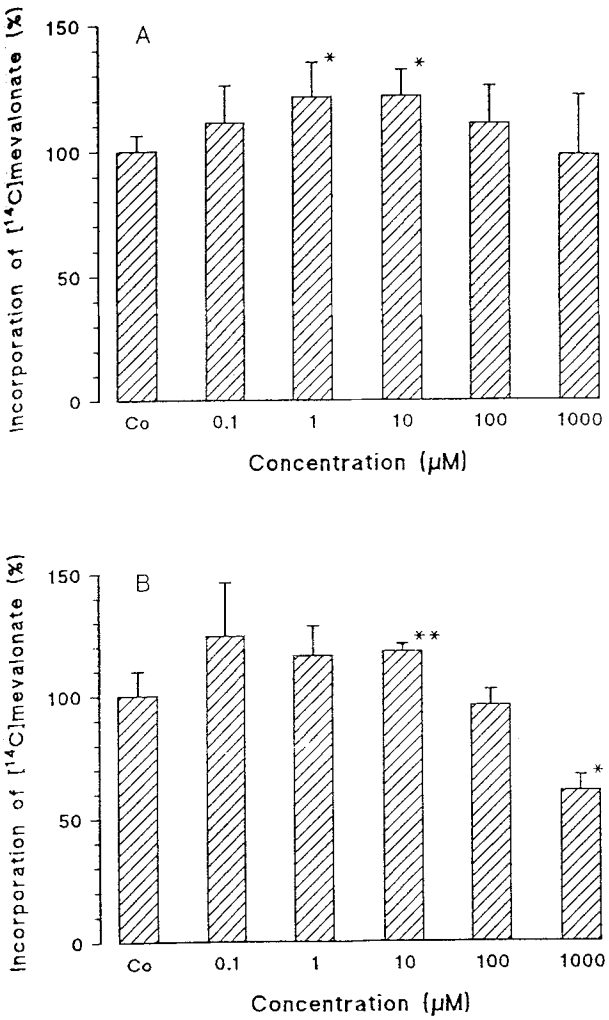


FIG. 4. Dependence of the incorporation of [^{14}C]mevalonate into non-saponifiable lipids of cultured rat hepatocytes on the concentration 1,3-vinyl-dithiin (A) and of 1,2-vinyl-dithiin (B). Data represent means ± SD of three to five independent determinations. Co, controls. *, Statistically different from controls, $P < 0.01$. **, Statistically different from controls, $P < 0.03$.

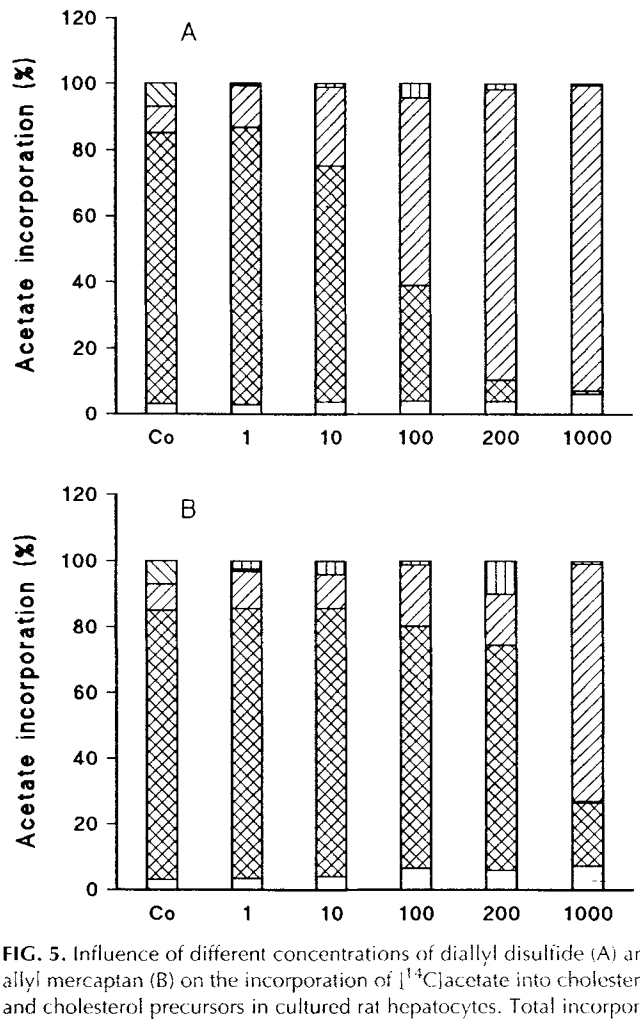


FIG. 5. Influence of different concentrations of diallyl disulfide (A) and allyl mercaptan (B) on the incorporation of [^{14}C]acetate into cholesterol and cholesterol precursors in cultured rat hepatocytes. Total incorporation was normalized to 100% for each concentration. Co, control; concentrations are given in μM. (crosshatched bar), cholesterol, (slanted-right lined bar), lanosterol, (slanted-left lined bar), 7-dehydrocholesterol, (vertical-lined bar), dihydrolanosterol, (open bar), other sterols.

lation of lanosterol at the expense of cholesterol and 7-dehydrocholesterol revealed considerable differences between these compounds (Fig. 6). Allicin was most effective with an EC_{50} value of 28 ± 4 μM (n=4) followed by diallyl disulfide with an EC_{50} value of 109 ± 14 μM (n=3). Thus, diallyl disulfide was about four times less potent than allicin. Diallyl disulfide, on the other hand, was approximately five times as potent as allyl mercaptan and 1,3-vinyl-dithiin which were almost equally effective (Fig. 6). 1,2-Vinyl-dithiin was completely ineffective and did not lead to the accumulation of lanosterol in the concentration range tested (Fig. 6).

When total incorporation of [^{14}C]acetate into cholesterol was determined, a similar sequence in the inhibitory potency of the compounds tested herein (allicin >> diallyl disulfide >> allyl mercaptan > 1,3-vinyl-dithiin and no influence of 1,2-vinyl-dithiin) was found (Fig. 7). Resulting from the different types of inhibition at early and late steps of the biosynthetic pathway demonstrated above, the quantitative effects were even more pronounced for allicin and dially disulfide than for the other compounds. Apparently, EC_{50} values amounted to 17 ± 2 μM (allicin), 64 ± 7 μM (diallyl disulfide), 450 ± 20 μM (allyl mercaptan), and > 600 μM (1,3-vinyl-dithiin) (n=4 for all compounds).

DISCUSSION

In the present study, several garlic-derived organosulfur compounds were compared for their ability to interfere with hepatocellular cholesterol biosynthesis *in toto* as well as during early and late reactions of the pathway. According to our results, the potential to inhibit incorporation of [^{14}C]acetate into cholesterol decreased in the order allicin >> diallyl disulfide >> allyl mercaptan > 1,3-vinyl dithiin, 1,2-vinyl dithiin being almost without effect. This inhibition results from different effects on early and late steps of cholesterol biosynthesis.

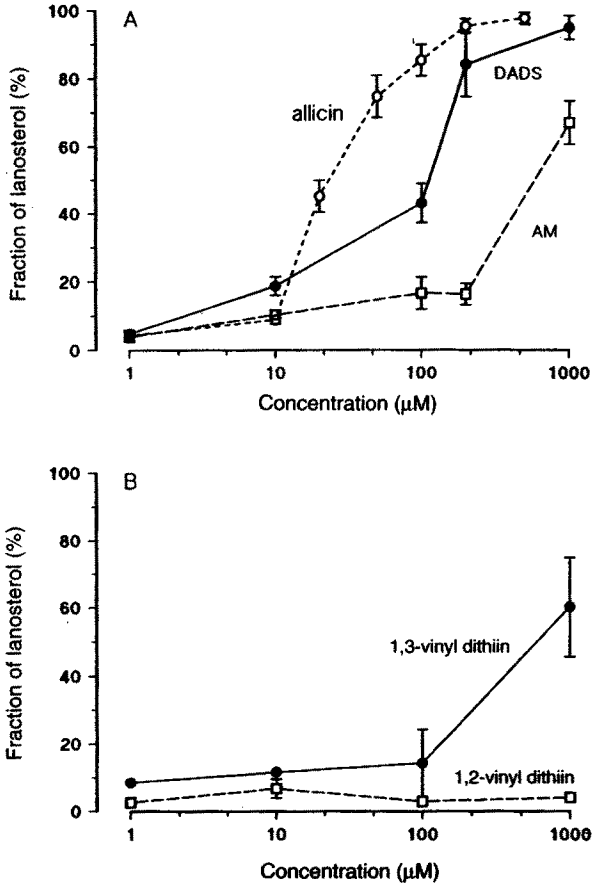


FIG. 6. Influence of different garlic-derived organosulfur compounds on the fraction of lanosterol among cholesterol and cholesterol precursors produced by cultured rat hepatocytes. (A) Concentration dependence of the influence exerted by allicin (○--○), diallyl disulfide (●—●), and allyl mercaptan (□···□). (B) Concentration dependence of the influence exerted by 1,3-vinyl-dithiin (●—●) and 1,2-vinyl-dithiin (□···□).

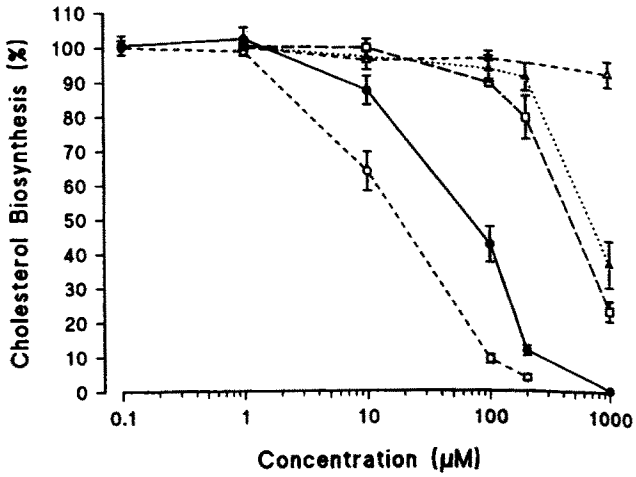


FIG. 7. Concentration dependence of the inhibition of cholesterol biosynthesis by different garlic-derived organosulfur compounds in cultured rat hepatocytes. Values are expressed as percentage of control cultures and represent means ± SD of three to four independent determinations. Allicin (○--○), diallyl disulfide (●—●), and allyl mercaptan (□···□), 1,3-vinyl-dithiin (▲···▲) and 1,2-vinyl-dithiin (△---△).

Concerning early steps of the pathway, the three compounds allicin, diallyl disulfide, and allyl mercaptan consistently inhibited the incorporation of [^{14}C]acetate into the fraction of neutral nonsaponifiable lipids which contained cholesterol, various sterol precursors, and squalene. In contrast to the results of Sendl *et al.* (17) who reported an enhanced incorporation for diallyl disulfide at 500 μM when using a liver homogenate assay system, no such enhancement was found in the intact cultured hepatocytes in the concentration range tested. When tested as early as 2 h after cultivation as done in this study, allicin was slightly more inhibitory than found recently for incubations starting after 18 h (22). Thus, the inhibitory potency of the three compounds at early steps decreased in the order allicin > diallyl disulfide = allyl mercaptan, the latter two compounds showing moderate effects only.

For allicin [this study and (22)] as well as for diallyl disulfide and allyl mercaptan, inhibition at the level of HMGCoA-reductase was apparent, because it disappeared if labeled acetate was replaced by labeled mevalonate. In the case of allicin, it has already been ruled out that the enzyme is directly inhibited in the respective concentration range (22). Whether this holds true also for diallyl disulfide and allyl mercaptan remains to be established, but there are first hints that these compounds might influence (decrease) the activity of HMG-CoA-reductase in an indirect manner through interactions with signal transduction pathways (21).

A second major result was the finding that the effects of the two vinyl-dithiins are qualitatively different from that of the other compounds tested. In contrast to all other garlic-derived organosulfur compounds tested herein and elsewhere (20,21) including allicin and ajoene (22), the vinyl-dithiins stimulated mevalonate incorporation into nonsaponifiable lipids at low concentrations between 1 and 100 μM. Only one isomer, the 1,2-vinyl-dithiin, caused inhibition at concentrations above 100 μM. At present there is no information on what enzymatic step might be stimulated in the lower concentration range. The fact that in the same concentration range incorporation of labeled acetate was not affected, i.e., remained within the variations of the controls, suggests that the vinyl-dithiins reduce the flux of acetate to mevalonate to an extent counterbalancing this stimulation. Preliminary experiments indicate that this reduction is exerted at the level of acetate uptake rather than on that of HMGCoA-reductase (Gebhardt, R., and Beck, H., unpublished observations). In this context, it is interesting to note that 1,2-vinyl-dithiin which is more lipophilic than 1,3-vinyl-dithiin (32) apparently was the more potent compound with respect to stimulation possibly due to enhanced binding to or solubilization within the plasma membrane. The two opposing effects of the vinyl-dithiins also might be responsible for the greater variations among different experiments of the data obtained with these compounds.

The third major point concerns the potential for inhibition of the cholesterol biosynthetic pathway at late steps involved in the conversion of the sterol precursors. Apparent from the

different accumulation of lanosterol, considerable differences between the organosulfur compounds tested herein also were observed for the inhibition at the level of lanosterol 14α-demethylase. As can be derived from the data presented, the inhibitory potential toward this enzymatic step decreased in the order allicin > diallyl disulfide > allyl mercaptan = 1,3-vinyl-dithiin >> 1,2-vinyl-dithiin, the latter being almost ineffective. It is obvious that inhibition at this step contributes most to the inhibition of cholesterol biosynthesis *in toto*. In addition, the accumulation of precursors due to the inhibition of lanosterol 14α-demethylase opens the possibility of an indirect inhibition of HMGCoA reductase similarly as described for other inhibitors of this enzyme (28).

Since diallyl disulfide and allyl mercaptan can occur as decomposition products of allicin in physiological buffers under certain conditions (33) or may be formed by the hepatocytes during metabolic conversion of allicin (34,35), it has been suggested that they might mediate the effects ascribed to allicin or other organosulfur compounds that might lead to their formation (2). This hypothesis can be ruled out at least partially, because of the lower sensitivity of the hepatocytes for diallyl disulfide and allyl mercaptan compared to that for allicin and the absence of any synergistic effects between diallyl disulfide and allyl mercaptan. Possibly, the common structural element of these compounds, i.e., the allylsulfide moiety, is responsible for their qualitatively similar effects. Their quantitatively different potency might be due to the adjacent structural elements similarly as has been described for the different reactivity with cysteine of the S-S bond in allicin, ajoene, and dially disulfide (2,36,37). Further studies on the structural requirements for the inhibitory action of these compounds are in progress.

Furthermore, the high concentrations of diallyl disulfide and allyl mercaptan necessary to exert any inhibition of acetate incorporation as found herein which are in the range of values published by other laboratories (16,17) render it rather doubtful that these compounds act directly by themselves, since such concentrations are unlikely to occur under physiological conditions. Thus, allicin which is at least four fold more effective might better fulfill the requirements for a definitive physiological effector, although its concentration in portal blood is not known and might be lowered by interaction with blood components (33).

On the other hand, in view of the low inhibitory potential of the vinyl-dithiins, conversion to more potent effectors may explain the findings obtained *in vivo* with oil-macerates (38). Although the vinyl-dithiins which are the predominant organosulfur compounds of oil-macerates (11,13,38,39) are relatively stable in blood (2,33), they might be sufficiently metabolized by the liver (32) to produce such effectors as diallyl disulfide or allyl mercaptan.

In conclusion, these results indicate that the influence of different garlic-derived organosulfur compounds on cholesterol biosynthesis varies considerably. Thus, it seems rather unlikely, that the observed hypocholesterolemic effect of garlic consumption may be mediated by some few (or even a single) compound(s) which are the most stable ones in the blood. Instead, one must assume that the liver may specifically sense many of the genuine or derived organosulfur compounds of garlic that are delivered to it *via* the portal vein after garlic consumption. The different stability of these compounds within the blood, however, may influence the spectrum of compounds acting on the liver and participating in the inhibition of cholesterol biosynthesis. In this respect, it is interesting that new and highly sensitive interactions with intracellular signalling pathways have been described for dially disulfide (21) or are under investigation as in the case of allicin (40, Gebhardt, R., unpublished observations).

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Biosynthesis of Eicosapentaenoic Acid in the Microalga *Porphyridium cruentum*. I: The Use of Externally Supplied Fatty Acids

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ABSTRACT: The biosynthetic pathways of eicosapentaenoic acid (20:5n-3) in microalgae, in general, and in *Porphyridium cruentum*, in particular, are not known. Some of the putative intermediates along the suggested pathways could not be detected probably due to their low endogenous level. In order to increase the endogenous levels of the intermediates, we provided various fatty acids in the growth medium. Exogenously supplied fatty acids were indeed incorporated into algal lipids and were further metabolized along the n-6 and n-3 pathways. In the n-6 pathway, 18:2 was desaturated to 18:3n-6, elongated to 20:3n-6, and subsequently desaturated to 20:4n-6 and then to 20:5n-3. In the n-3 pathway, 18:2 was first desaturated to 18:3n-3 which was then sequentially converted, apparently by the same enzymatic sequence of the n-6 pathway to 18:4n-3, 20:4n-3, and 20:5n-3.

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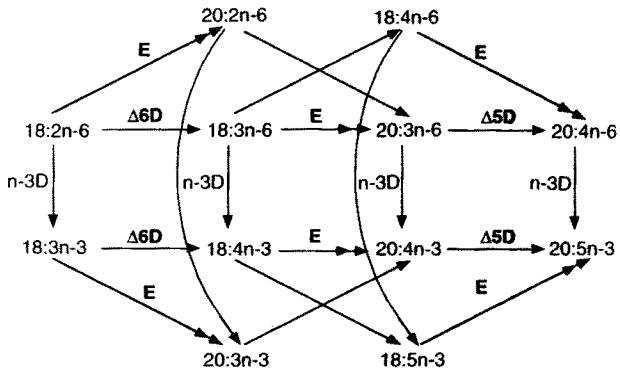


FIG. 1. Theoretically possible pathways of eicosapentaenoic acid biosynthesis in *Porphyridium cruentum*. Δ6D represents the Δ6 desaturase; E- the elongation system; Δ5D- the Δ5 desaturase; and n-3D- the n-3 desaturase.

The recent interest in the pharmacological properties of eicosapentaenoic acid (EPA) (20:5n-3) prompted the search for alternative sources for these fatty acids, such as algae (1) and fungi (2), to replace the commonly used marine fish oil. *Porphyridium cruentum* is one of the promising sources (3). In order to reach contents which will allow economical production, genetic methods must be eventually employed. This in turn requires a comprehensive understanding of the biosynthetic pathways of EPA in terms of intermediates and enzymes. The biosynthetic pathways leading to polyunsaturated fatty acids (PUFA) in leaves of higher plants were recently reviewed by Browse and Somerville (4). Except for the first double bond, desaturation involves lipid-linked fatty acids. The biosynthesis of algal PUFA is presumably similar to that of higher plants at least to the level of 18:2n-6. However, there could be 12 different theoretical pathways, at the fatty acid level, by which 18:2n-6 can be converted to 20:5n-3 (Fig. 1). One of the impediments to the elucidation of the biosynthesis of EPA is that some of the putative intermediates may be present at very low levels which makes detection difficult. Incorporation of radiolabeled precursors would give only partial results since the identification of the various molecular species of individual lipids would be extremely difficult without molecular species standards. A possible approach for increasing the endogenous content of a putative metabolite is the feeding of algal cells with exogenously supplied fatty acid intermediates (5–9). We have speculated that the incorporated fatty acid intermediates would elevate their endogenous levels and that of subsequent metabolites along the biosynthetic routes. The results of the feeding experiments indicate that most likely there are only two pathways leading to the production of EPA.

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Abbreviations: C_n fatty acids, fatty acids with n carbon atoms; EPA, eicosapentaenoic acid (20:5n-3); GC, gas chromatography; MGDG, monogalactosyldiacylglycerol; PUFA, polyunsaturated fatty acids; X/Y, a fatty acyl group containing X carbon atoms and Y double bonds (*cis*). Pairs of numbers representing the fatty acids, when separated by a slash, designate the components in the *sn*-1 and *sn*-2 positions, respectively, of the molecular species. n-x indicates that the last double bond in a methylene-interrupted PUFA is n carbon atoms removed from the end of the chain. Δn indicates a double bond at the nth carbon atom. ΔnD indicates a desaturase that introduces a double bond at the nth carbon atom.

MATERIALS AND METHODS

Organism and culture conditions. Porphyridium cruentum strain 1380.1d was obtained from the Göettingen Algal Cul-

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ture Collection (Göettingen, Germany) and was grown on Jones' medium (11) as previously described (12) in Erlenmeyer flasks under an air–CO_2 (99:1) atmosphere at 25°C. The flasks were placed in an incubator shaker and illuminated from above at 115 μmol quanta m^{-2} s^{-1}. Cultures were grown exponentially (with proper dilution) for at least 4 d prior to the onset of the experiment and were diluted daily throughout the experiment. Solutions of fatty acids in dimethylsulfoxide were added to a final concentration of 0.17 mM. The dimethylsulfoxide concentration in the treated and control cultures didn't exceed 1%.

Extraction and fractionation of lipids. Freeze-dried samples of algal biomass were extracted by grinding with sand in chloroform/methanol/water (2:1:0.8) according to Bligh and Dyer (13). Separation into neutral lipids, galactolipids, and phospholipids fractions was performed with a silica gel cartridge (Sep-pak; Waters Division of Millipore, Milford, MA); the individual fractions were eluted successively with chloroform, acetone, and methanol, respectively. The galactolipid fraction was further separated by thin-layer chromatography on silica gel plates and developed with chloroform/acetone/methanol/acetic acid/water (100:40:20:20:10, by vol).

Separation, identification, and quantitation of molecular species of monogalactosyldiacylglycerol (MGDG) was performed by high-performance liquid chromatography on Lichrocart 250-4 (RP-18, 5 μ) cartridges (Merck, Darmstadt, Germany) by elution with 93% methanol/water at a flow rate of 2 mL min^{-1}. Peaks were detected at 205 nm, isolated, and transmethylated as described below. The fatty acid composition was elucidated by gas chromatography (GC) analysis. For quantitation of the HPLC chromatogram, we have used an evaporative light-scattering detector (ELSD-2; Varex, Burtonsville, MD). The settings of the detector were: tube temperature 130°C, nitrogen flow rate 40 mL min^{-1}.

Fatty acid analysis. Lipids were transmethylated with methanol–acetyl chloride and analyzed by GC as previously described (14). Fatty acid methyl esters were identified by comparison of their equivalent chain length (15), by cochromatography with authentic standards (Sigma Chemical Co., St. Louis, MO and *Mortierella alpina* biomass) and by GC–mass spectrometry (12). The data shown represent mean values with a range of less than 5% for major (over 10% of fatty acids) peaks and 15% for minor peaks, of four independent samples, each analyzed in duplicate.

RESULTS AND DISCUSSION

The fatty acid profile of *P. cruentum* under control conditions was composed of 18:2, 18:3n-6, 20:3n-6, 20:4n-6, and 20:5n-3 (Table 1). The appearance of fatty acids of the n-6 family point to the existence of an n-6 pathway from 18:2 to 20:4n-6 as detailed in Sequence 1. Fatty acids which could indicate the existence of the n-3 pathway that is shown in Sequence 2 were not detected except for 20:5n-3.

$$18{:}2n\text{-}6_{9,12} \xrightarrow{\Delta 6D} 18{:}3n\text{-}6_{6,9,12} \longrightarrow 20{:}3n\text{-}6_{8,11,14} \xrightarrow{\Delta 5D} 20{:}4n\text{-}6_{5,8,11,14} \quad [1]$$

$$18{:}2n\text{-}6_{9,12} \xrightarrow{\Delta 15D} 18{:}3n\text{-}6_{9,12,15} \xrightarrow{\Delta 6D} 18{:}4n\text{-}3_{6,9,12,15} \longrightarrow 20{:}4n\text{-}3_{8,11,14,17} \xrightarrow{\Delta 5D} 20{:}5n\text{-}3_{5,8,11,14,17} \quad [2]$$

TABLE 1
Fatty Acid Composition of the Lipids of *Porphyridium cruentum* Following Incorporation of PUFA of the n-6 Family

Fatty acid added	Lipid analyzed	Fatty acid composition (% of total fatty acids)														
		16:0	16:1 n-11	16:1[a]	16:3[a]	18:0	18:1 n-9	18:2 n-6	18:3 n-6	18:3 n-3	20:2 n-6	20:3 n-6	20:3[b] Δ5	20:4 n-6	20:4 n-3	20:4[c] Δ5	20:5 n-3
None	Total lipids	30.3	3.3	3.1	—	0.5	0.5	5.3	0.7	—	0.4	0.6	—	16.8	—	—	37.9
18:2n-6		25.7	3.6	1.9	0.4	0.3	0.5	15.8	0.6	0.3	2.7	1.2	0.2	23.2	0.4	—	22.9
18:3n-6		24.0	7.2	2.2	—	0.8	0.9	5.7	3.6	0.3	0.3	1.0	—	18.8	—	—	34.6
20:3n-6		28.0	4.3	2.9	0.1	0.4	0.5	5.0	0.6	—	0.6	7.1	—	15.0	—	—	35.2
20:2n-6		27.3	3.7	—	0.2	0.3	0.6	5.1	0.8	—	11.7	0.5	1.9	10.4	—	1.5	32.6
None	NL	22.6	0.4	0.8	0.5	2.6	1.9	22.2	1.6	—	1.4	1.4	—	28.3	—	—	18.4
18:2n-6		12.8	0.7	0.6	—	0.9	1.3	39.4	2.5	0.4	3.3	1.6	—	26.2	0.5	—	10.0
18:3n-6		24.3	—	—	0.4	5.1	3.0	18.1	10.5	0.5	1.7	2.4	—	24.8	—	—	6.4
20:3n-6		11.8	51.0	0.1	1.4	1.9	0.6	10.3	1.4	0.3	0.8	5.3		10.6	—	—	4.5
20:2n-6		3.7	12.6	0.1	2.2	0.7	0.4	42.2	0.1	0.1	30.0	0.2	—	3.8	—	0.2	3.5
None	GL	32.4	0.3	0.7	—	0.4	0.5	4.7	0.1	—	0.4	0.2	—	7.1	—	—	53.1
18:2n-6		26.4	6.3	0.3	0.7	0.4	0.5	14.2	0.3	0.4	1.9	0.4	0.1	17.9	—	—	30.0
18:3n-6		22.6	11.9	0.1	—	0.7	0.8	5.8	5.2	—	—	0.5	—	7.6	—	—	44.9
20:3n-6		32.7	0.8	0.4	—	0.4	0.5	4.5	0.1	—	0.8	5.3	—	6.6	—	—	47.7
20:2n-6		29.3	0.2	0.1	0.1	0.3	0.5	4.6	0.1	—	9.5	0.2	—	5.0	—	2.3	47.6
None	PL	25.2	—	9.4	—	0.4	0.6	4.9	2.0	—	0.3	1.4	—	35.3	—	—	20.0
18:2n-6		25.2	0.4	5.5	1.3	2.1	1.1	8.2	0.4	0.2	3.3	2.1	0.2	29.4	3.8	—	15.5
18:3n-6		26.9	0.4	7.5	—	0.5	0.8	3.9	2.3	—	0.2	1.7	—	36.5	—	—	18.6
20:3n-6		27.6	0.2	10.2	—	0.5	0.7	5.3	1.5	—	0.5	2.1	—	31.5	—	—	18.6
20:2n-6		31.1	0.1	8.6	—	0.4	0.7	4.8	1.6	—	5.7	0.6	3.7	16.9	—	1.1	24.2

[a]Unidentified isomer. [b]20:3(5,11,14). [c]20:4(5,11,14,17). NL, neutral lipids; GL, galactolipids; PL, phospholipids; PUFA, polyunsaturated fatty acids.

where ΔnD indicates a desaturase that introduces a double bond at the nth carbon atom.

The failure to detect intermediates of the putative n-3 pathway could have resulted from one of two reasons (i) the n-3 pathway does not exist in this alga or (ii) the n-3 pathway does exist, but the endogenous level of the intermediates was too low for detection. However, the mere existence of certain fatty acids does not in itself prove that they are indeed intermediates in any pathway of PUFA biosynthesis. The conversion of 18:2 to 20:5 involves one elongation and three desaturation steps. Theoretically, there are at least 12 possible routes, at the fatty acid level, as shown in Figure 1. Indeed, Arao and Yamada (16) suggested that 4 pathways are operative for this conversion in *Phaeodactylum tricornutum*. A pathway in which the elongation of 18:2n-6 to 20:2n-6 precedes further desaturation was claimed to take place in *Euglena* (17).

The conversion of C_{18} to C_{20} PUFA requires one more elongation step. Since elongation occurs at the carboxyl end, the fatty acid to be elongated must first detach from the lipid where it resides. These detaching and reattaching steps which are not found in the biosynthetic pathways of C_{18} PUFA suggest that externally introduced fatty acids also could be incorporated into polar lipids. Thus, we hypothesized that a possible means to increase the endogenous levels of putative intermediates was by supplementing the growth medium with fatty acids which were assumed to participate in either pathway. We also hoped to be able to show precursor–product relationship between the putative intermediates.

Cultures of *P. cruentum* were thus fed with various C_{18} and C_{20} PUFA. After 3 d, the lipids were extracted and separated to the individual lipid classes and analyzed. All the externally introduced fatty acids were indeed incorporated into the lipids and were accumulated at proportions far exceeding those which could be observed under control conditions.

Incorporation of 18:2n-6. Growing *P. cruentum* on a medium supplemented with 18:2n-6, a branching point for the putative n-3 and n-6 pathways (Fig. 1) resulted as expected in a 2–5-fold increase in its content in all lipid fractions (Table 1). The level of subsequent intermediates of the n-6 pathway in neutral lipids and galactolipids also increased but to a much lesser extent. In the fraction of the phospholipids, the pattern was not consistent since some intermediates increased while others decreased. While putative intermediates of the n-3 family could not be detected in control cultures, they were detected in all lipid fractions following growth of the alga in the presence of 18:2n-6. The proportion of 18:3n-3 reached 0.4% in neutral and galactolipids and 0.2% in phospholipids. Furthermore, 20:4n-3 appeared in neutral and phospholipids at 0.5 and 3.8%, respectively. These data clearly indicate the ability of the algal cells to convert 18:2n-6 to 18:3n-3 by n-3 desaturase(s) and to further convert it by subsequent elongation and desaturation steps to 20:4n-3.

Incorporation of possible intermediates of the n-3 family. According to Figure 1 and the results shown in Table 1, 18:3n-3 is the immediate product of the desaturation of 18:2n-6 by n-3 (Δ15) desaturase(s), and it is the first intermediate in the n-3 pathway. In order to find out whether the appearance of other n-3 pathway intermediates after feeding with 18:2n-6 supports the n-3 pathway, we fed the alga with 18:3n-3. As can be seen from Table 2, all the fatty acids of sequence 2, as well as 20:3n-3 were detected. Thus, 18:3n-3 could be further converted to 20:4n-3 either *via* 18:4n-3, 20:3n-3, or both (Sequence 3).

TABLE 2
Fatty Acid Composition of the Lipids of *Porphyridium cruentum* Following Incorporation of PUFA of the n-3 Family

		Fatty acid composition (% of total fatty acids)																
Fatty acid added	Lipid analyzed	16:0	16:1 n-11	16:1[a]	16:3[a]	18:0	18:1 n-9	18:2 n-6	18:3 n-6	18:3 n-3	18:4 n-3	20:2 n-6	20:3 n-6	20:3 n-3	20:4 n-6	20:4 n-3	20:4[b] Δ5	20:5 n-3
None	Total lipids	30.3	3.3	3.1	—	0.5	0.5	5.3	0.7	—	—	0.4	0.6	—	16.8	—	—	37.9
18:3n-3		18.9	0.9	2.6	0.1	0.2	0.6	5.6	1.0	18.0	1.9	0.5	0.5	0.7	10.6	0.3	—	36.4
18:4n-3		12.8	0.9	0.8	0.4	0.9	1.2	5.6	0.3	0.1	24.3	—	0.9	—	6.1	14.8	—	30.8
20:3n-3		26.5	1.5	3.5	0.5	0.3	0.4	5.0	0.5	0.7	—	0.4	0.6	4.0	14.5	—	9.0	32.4
None	NL	22.6	0.4	0.8	0.5	2.6	1.9	22.2	1.6	—	—	1.4	1.4	—	28.3	—	—	18.4
18:3n-3		6.6	0.2	—	0.2	0.5	1.1	13.0	1.3	40.0	2.5	0.3	1.3	0.4	13.9	1.1	trace	13.9
18:4n-3		10.6	1.0	0.4	0.6	0.6	1.0	5.5	0.3	—	29.5	2.7	0.8	—	3.2	12.1	—	31.3
20:3n-3		26.4	0.2	0.9	1.3	3.5	0.9	12.1	1.7	0.8	—	0.6	0.9	15.1	15.7	—	4.7	15.2
None	GL	32.4	0.3	0.7	—	0.4	0.5	4.7	0.1	—	—	0.4	0.2	—	7.1	—	—	53.1
18:3n-3		27.3	4.3	0.2	0.9	0.4	0.8	4.6	0.2	11.6	0.5	0.5	0.1	0.8	3.9	0.3		42.6
18:4n-3		29.1	—	—	—	0.8	1.0	5.2	0.1	—	5.3	—	0.2	—	1.1	4.5	—	52.8
20:3n-3		30.6	0.4	0.9	0.1	0.5	0.4	4.7	0.1	0.4	—	0.5	0.2	3.9	7.3	—	9.2	40.8
None	PL	25.2	—	9.4	—	0.4	0.6	4.9	2.0	—	—	0.3	1.4	—	35.3	—	—	20.0
18:3n-3		28.9	0.2	7.4	—	0.5	1.1	4.7	1.3	8.8	1.7	0.9	0.9	1.4	15.4	0.4	—	24.8
18:4n-3		23.0	0.4	3.8	—	1.0	0.9	2.1	0.6	—	6.8	—	3.2	—	12.8	16.5	—	28.3
20:3n-3		23.7	0.4	8.4	—	0.6	1.0	4.2	1.1	1.5	—	0.5	1.1	3.5	27.8	—	8.6	17.0

[a]Unidentified isomer. [b]20:4(5,11,14,17). NL, neutral lipids; GL, galactolipids; PL, phospholipids; PUFA, polyunsaturated fatty acids.

$$\begin{array}{ccc} 18{:}3n\text{-}3 & \xrightarrow{\Delta 6D} & 18{:}4n\text{-}3 \\ n\text{-}3D\downarrow & & n\text{-}3D\downarrow \\ 20{:}3n\text{-}3 & \xrightarrow{\Delta 6D} & 20{:}4n\text{-}3 \end{array} \quad [3]$$

In order to determine which of the various routes in Sequence 3 is the one leading to the production of 20:4n-3 and perhaps to 20:5n-3, we provided the alga with 18:4n-3. Both the 18:4n-3 acid and its elongation product 20:4n-3 were incorporated into all lipids fractions. The latter was mainly found in neutral lipids (12.1%) and phospholipids (16.5%) (Table 2). These findings are in keeping with Sequence 2 and suggest that this pathway includes 18:4n-3 rather than 20:3n-3.

Incorporation of 20:3n-3 resulted in accumulation of this fatty acid in all lipid classes but especially in neutral lipids (Table 2). However, we could not detect even traces of 20:4(8,11,14,17) supporting our conclusion that in the postulated n-3 pathway, 18:3n-3, rather than 20:3n-3, is the precursor for 20:4n-3 (Sequence 2) and that *P. cruentum* is incapable of a Δ8 desaturation (see below). Furthermore, we have observed the appearance of 20:4(5,11,14,17) (20:4Δ5) which was identified, by comparison to authentic samples and by GC–mass spectrometry. (The significance of the appearance of nonmethylene-interrupted PUFA with respect to the specificity of the desaturases will be addressed elsewhere.)

Incorporation of possible intermediates of the n-6 family. The detection of intermediates of the n-6 pathway in control cells (Table 1) is indicative of the existence of this pathway (Sequence 1). The increase in the proportions of 20:3n-6 and 20:4n-6 following the growth of the cells in the presence of 18:3n-6 further supports this conclusion (Table 1). No fatty acids of the n-3 series (except for EPA) could be detected following growth of the alga in the presence of either 18:3n-6 or 20:3n-6 (Table 1). We thus infer that neither 18:3n-6 nor 20:3n-6 is likely substrate for an n-3 desaturation.

According to Figure 1, 20:2n-6 could be converted by a Δ8D to 20:3n-6 or by an n-3D to 20:3n-3. When provided to the cells, this fatty acid was incorporated into neutral lipids and phospholipids (Table 1). However, there was no enhancement in the level of any other fatty acid in either the n-6 or n-3 pathways while EPA actually decreased, indicating that the 20:2n-6 was not an intermediate in either pathway. Unexpectedly, we observed the appearance of 20:3(5,11,14) (20:3Δ5) and 20:4Δ5 (see above) which further supports our hypothesis that the Δ8 double bond can not be introduced to C_{20} fatty acids. Rather, a Δ6 double bond is introduced into a C_{18} fatty acid which upon elongation becomes the Δ8 double bond.

The conversion of 18:2 to EPA requires one n-3 desaturation step. Theoretically, four n-6 intermediates could be used as substrate for the n-3 desaturation, namely, 18:2n-6, 18:3n-6, 20:3n-6, and 20:4n-6 (Fig. 1). However, the data we have obtained suggest that only 18:2 (Table 1) and 20:4n-6 (see below) are desaturated to 18:3n-3 and 20:5n-3, respectively. These findings raise several questions that require further study: (i) Are both desaturations carried out by one enzyme which has broad substrate specificity or by two enzymes with specificity toward 18:2 and 20:4, respectively? (ii) If indeed two desaturases are involved and knowing that the 20:4 desaturase is apparently residing in the chloroplast, is the 18:2 desaturase located in the chloroplast or in the cytoplasm?

In similarity to the n-6 intermediates, those of the n-3 pathway, namely 18:4n-3 and 20:4n-3, were primarily concentrated in the phospholipid and the neutral lipid fractions (Table 2). This finding suggests that the Δ6 and Δ5 desaturation steps in both pathways are cytoplasmic, utilizing phospholipids as their acyl carriers. Recently, using radiolabled precursors, we have shown that the Δ6D and the Δ5D of both the n-3 and the n-6 pathways utilize phosphatidylcholine-bound fatty acids (18). Thus, we speculate that both pathways utilize the same Δ6D and the Δ5D activities.

Putative products of the Δ6 desaturase, 18:4n-3, and 18:3n-6 in the n-3 and n-6 pathways, respectively, produced seemingly unrelated phenomena. Incorporation of 18:4n-3 resulted in a surprising substantial decrease (up to 85%) in the proportion of n-6 fatty acids in every lipid class analyzed (Table 2). When either 18:3n-6 or 20:3n-6 was incorporated, we observed the appearance of 18:3n-3 which normally is not detectable (Table 1). We propose that these findings could be explained by inhibition of the Δ6 desaturation that is brought about by elevated 18:4n-3 or 18:3n-6 (Sequence 1 and 2). The decrease in the Δ6 desaturase activity caused by 18:4n-3 also would be manifested in the reduction in the level of 18:3n-6 and its metabolites in the n-6 pathway (Sequence 1), and eventually in a decrease in 20:4n-6. Similarly, inhibition of the Δ6 desaturation by 18:3n-6 led to an accumulation of 18:2 and/or 18:3n-3. The elevated level of 18:3n-3 also could result from the increased availability of 18:2 to the n-3 desaturase. In any event, the appearance of 18:3n-3 is additional indication for the existence of the first step of the n-3 pathway, namely, the conversion of 18:2 to 18:3n-3 (Sequence 2) which seems to be the rate-limiting one in the n-3 pathway.

Since one could not rule out the possibility that the pathways revealed by the feeding experiments are not operational in the absence of exogenously supplied fatty acids, we have conducted several studies using radiolabeled precursors (18). The data we have obtained supported our two-pathway hypothesis.

Molecular species of monogalactosyldiacylglycerol (MGDG). MGDG can be divided into prokaryotic and eukaryotic molecular species according to the positional distribution of fatty acid substituents. In higher plants, these molecular species are named after the different pathways from which they evolve. Thus, we attempted to determine whether the changes observed in the fatty acid profile following the incorporation of externally provided fatty acids could be attributed to all or particular molecular species.

We have shown above (Table 1) that exogenously added 18:2 increased the level of 20:4n-6 at the expense of EPA. Analysis of the molecular species of MGDG has further demonstrated a sharp increase in the proportion of

20:4n-6/20:5n-3 and 20:4n-6/16:0 and a decrease in that of 20:5n-3/20:5n-3 and 20:5n-3/16:0 (Table 3). These findings may suggest the existence of precursor–product relationship between the former and the latter in keeping with Sequences 4 and 5. Furthermore, these findings indicate that the chloroplast is the site of the n-3 desaturation of both the eukaryotic and prokaryotic species of MGDG.

$$20{:}4n\text{-}6/16{:}0 \longrightarrow 20{:}5n\text{-}3/16{:}0 \quad [4]$$

$$20{:}4n\text{-}6/20{:}4n\text{-}6 \longrightarrow 20{:}4n\text{-}6/20{:}5n\text{-}3 \longrightarrow 20{:}5n\text{-}3/20{:}5n\text{-}3 \quad [5]$$

Support to the above conclusion was recently obtained using radiolabeled precursors (16). We have found that (i) in *P. cruentum*, the diacylglycerol 20:4n-6/20:4n-6 which participates in the eukaryotic pathway is, most likely, imported from the cytoplasm, and following galactosylation, is sequentially desaturated to 20:5n-3/20:5n-3 MGDG and (ii) the prokaryotic molecular species 20:4n-6/16:0 is also desaturated to 20:5n-3/16:0.

We have also observed the appearance of the eukaryotic-like species 20:4n-6/18:2 (7.4% of MGDG) (Table 3) which was not detected in the control culture. The appearance of "eukaryotic-like" molecular species of MGDG in the prokaryotic organism, *Spirulina,* was recently reported by Pham Quoc *et al.* (10). They have shown that an externally supplied C_{18} fatty acid was incorporated into the *sn*-2 position producing C_{18}/C_{18} "eukaryotic-like" species, partially replacing the common prokaryotic species C_{18}/C_{16}. Thus, the appearance of "eukaryotic-like" species 20:4n-6/18:2 in *P. cruentum* is analogous to the C_{18}/C_{18} "eukaryotic-like" species of *Spirulina.*

Following the addition of 18:3n-3 (Table 3), we have observed the appearance of 18:3n-3/16:0 (6.8% of total molecular species) as well as a low level (0.6%) of the eukaryotic-like species 20:5/18:3n-3. It appears that externally added 18:2 and 18:3n-3 were preferentially incorporated into different positions, the *sn*-2 and *sn*-1 positions of MGDG, respectively, a phenomenon which we can not explain at the moment. In the presence of 18:4n-3, there was an almost complete elimination of all 20:4n-6-containing molecular species (Table 3), further supporting our hypothesis that 18:4n-3 inhibits Δ6 desaturation (see above). As a result of this inhibition, all C_{20}-containing molecular species were produced *via* the n-3 pathway. We have also observed a decrease from 44.2 to 32.7% in the prokaryotic 20:5/16:0 and a concurrent increase from 36.7 to 57.3% in the eukaryotic 20:5/20:5. This may indicate that 18:4n-3 and hence the n-3 pathway in which it participates is preferentially involved in the production of eukaryotic species of MGDG. Addition of 20:3n-3, which is not an intermediate in the n-3 pathway (see above), resulted in a reduction in the major eukaryotic product 20:5/20:5 (Table 3) and in the appearance of 20:5/20:4(5,11,14,17) which could result from the cytoplasmic Δ5 desaturation of 20:5/20:3n-3.

TABLE 3
Effect of Exogenously Supplied Fatty Acids to *Porphyridium cruentum* on the Molecular Species Composition of MGDG

Molecular species of MGDG	Fatty acid supplied				
	None	18:2	18:3n-3	18:4n-3	20:3n-3
	Molecular species (% of total)				
20:5/20:5	36.7	19.4	41.6	57.3	22.4
20:5/18:3n-3	—	—	0.6	—	—
20:5/20:4n-3	—	—	—	2.2	—
20:5/20:4Δ5[a]	—	—	—	—	4.3
20:5/20:4n-6	6.4	19.7	3.2	trace	4.2
X[b]	—	5.9	—	—	—
20:4n-6/18:2	—	7.4	—	—	—
20:5/16:0	44.2	25.2	41.8	32.7	46.3
18:3n-3/16:0	—	—	6.8	—	—
20:4Δ5[a]/16:0	—	—	—	—	11.9
20:4n-6/16:0	3.5	14.3	10.0	trace	trace
18:2/16:0	9.1	8.1	4.3	7.8	10.9

[a]20:4(5,11,14,17); MGDG, monogalactosyldiacylglycerol.
[b]Unidentified.

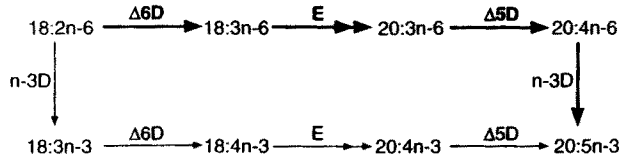


FIG. 2. Outline of the n-3 and n-6 pathways in the biosynthesis of eicosapentaenoic acid in *Porphyridium cruentum.* The dominant pathway is shown in bold. For legend see Figure 1.

Conclusions. Our data suggest that EPA in *P. cruentum* is most likely synthesized along the n-6 and n-3 pathways as presented in Figure 2.

The 18:4n-3 and 18:2 acids inhibit the Δ6 and n-3 desaturations, respectively, and could possibly be used as inhibitors for various steps in the biosynthesis of C_{20} and C_{22} PUFA.

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The Fatty Acid Composition of Human Gliomas Differs from That Found in Nonmalignant Brain Tissue

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ABSTRACT: To compare the fatty acid composition of tumor tissue from glioma patients with that of normal brain tissue, tissue samples were obtained from 13 glioma patients and from 3 nonmalignant patients. Following lipid extraction, total fatty acid composition was measured using gas–liquid chromatography. Samples were further separated into phospholipids and neutral lipids. Representative samples were then separated into phospholipid classes by thin-layer chromatography and the fatty acid composition assayed. Levels of the polyunsaturated fatty acid (PUFA) docosahexaenoic acid (DHA), were significantly reduced ($P = 0.029$) in the glioma samples compared with normal brain samples; mean values were 4.8 ± 2.9% and 9.2 ± 1.0%, respectively. This reduction in glioma DHA content was also observed in terms of phospholipids (4.6 ± 2.1% vs. 9.6 ± 0.8%, $P = 0.002$). The phosphatidylserine and phosphatidylethanolamine phospholipid classes were reduced in the glioma samples. Differences were also noted in the n-6 PUFA content between glioma and normal brain samples. The glioma content of the n-6 PUFA linoleic acid was significantly greater ($P < 0.05$) than that observed in the control samples in terms of total lipids. Thus, the fatty acid composition of human gliomas differs from that found in nonmalignant brain tissue.

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In 1995, there will be an estimated 17,200 new cases of central nervous system (CNS) tumors and 13,300 deaths from CNS tumors in the United States (1). The majority of these will arise from malignant gliomas of the brain (approximately 8500 new cases and 6600 deaths estimated for 1995) (2). Treatment results for malignant gliomas remain poor, especially for the most common and most aggressive glioma, glioblastoma multiforme (GBM). Despite multimodality therapy including surgery, radiation therapy, and chemotherapy, the median survival for GBM remains ≤1 yr, and long-term survival rates are dismal (3). Many different therapeutic approaches have been studied in an attempt to improve the outcome of patients with malignant gliomas, including altered forms or fractionation of radiotherapy, brachyradiotherapy, radiosensitizers, and various chemotherapeutic agents. At best, these attempts have provided modest benefit.

An alternative approach might be the use of polyunsaturated fatty acids (PUFA). Fatty acids in tissues are derived from either the diet or through biosynthesis. Saturated and monounsaturated fatty acids can be synthesized *de novo.* If sufficient amounts are not available in the diet, synthesis is increased to maintain an adequate supply. However, the n-3 and n-6 PUFA cannot be synthesized *de novo* and are derived entirely from the diet. Thus, tissue PUFA content will depend to a large extent on the diet; tissue PUFA composition will change if the diet is PUFA-deficient or PUFA-rich. The same principle applies to malignant cells, which derive all their PUFA from the host. *In vitro* data indicate that tumor cells are deficient in PUFA as compared with their normal cell counterparts (4,5). Increasing the PUFA content of tumor cells *in vitro* by incubating the cells in media supplemented with PUFA selectively potentiates tumor cell sensitivity to subsequent therapies involving oxidative stress (6); normal cells are relatively unaffected. The mechanism of this cytotoxic effect appears to be *via* enhanced free radical generation and lipid peroxidation. A reduced PUFA content also has been observed *in vivo* in tumor samples obtained from prostatic (7) and breast cancer patients (8). Moreover, increasing malignant cell PUFA content by dietary supplementation has been shown to inhibit tumor growth in nude mice implanted with human breast cancer (9) and prostatic cancer cells (10).

The purpose of the present study was to determine the fatty acid composition of human glioma tumors and compare this with the fatty acid composition of normal brain tissue. A reduction in the PUFA content of glioma cells relative to normal brain tissue could contribute to the relative resistance of gliomas to therapy. This might provide the basis for studies to determine the potential role of PUFA as adjunct agents in the radiotherapeutic treatment of gliomas.

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Abbreviations: ALA, α-linolenic acid; CNS, central nervous system; DHA, docosahexaenoic acid; GBM, glioblastoma multiforme; GLC, gas–liquid chromatography; LA, linoleic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; UI, unsaturation index.

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MATERIALS AND METHODS

Patients and samples. Scientific study of resected brain tissue was approved through the institutional human use committee. Brain tissue samples were collected between February 1994 and December 1994 from 13 patients with glioma tumors and 3 control patients who were undergoing brain tissue resection for seizure disorder. Information concerning the glioma patients and the control patients are shown in Tables 1 and 2, respectively.

Tissue sample collection and lipid extraction. Fresh tissue (50 mg–1 g) was collected in the operating room and placed on ice within 10 min of resection. Any blood present was removed by washing with chilled normal saline, and the sample was then placed in a chloroform/methanol mixture ($CHCl_3$–CH_3OH; 2:1, vol/vol) and stored at −20°C until analyzed. The lipids were extracted according to the method of Folch *et al.* (11). Aliquots of lipid extract from each patient sample were separated into phospholipid and neutral lipid fractions using silicic acid column separation. Aliquots of the phospholipid fraction were separated by thin-layer chromatography into phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylinositol (PI) fractions (12).

Fatty acid composition. Fatty acid composition was determined by gas–liquid chromatography (GLC) (12). Briefly, the fatty acid composition of each patient sample was determined in terms of the total lipid, neutral lipid, total phospholipid, and PE, PC, PS, and PI fractions. To a volume of each lipid extract, a known amount of heptadecaenoic acid methyl ester (17:0) was added as an internal standard. The fatty acids were then methylated for 50 min at 95°C with 12% BF_3 (12) and separated by GLC using a 1.9-m column packed with SP-2330 on Chromosorb Gaschrome WAW (Supelco, Bellefonte, PA). The resultant peaks were identified by comparing the retention times with those of known fatty acid methyl ester standards. Peak areas were determined by automatic integration.

Statistical analysis. Data are expressed in terms of mean ± SD. The unpaired Student's two-tailed *t*-test was used to assess the differences in mean fatty acid levels and unsaturation indices between tumor and control sample populations.

TABLE 1
Study Patient Data[a]

Patient number	Age at Dx (yr)	Sex	Location of lesion	Diagnosis	Associated conditions	Tx before surgery	Tx after surgery	Status (mon after surgery)
1	3	F	Thalamus	Pilocytic Astrocytoma	none	none	Radiotherapy	NED 16 mon
2	13	M	Rt cerebellum	Subependymal Giant Cell Astrocytoma	Tuberous Sclerosis	none	none	NED 12 mon
3	29	F	Rt frontal lobe	Oligodendroglioma	none	none	none	NED 6 mon
4	36	M	Rt parietal lobe	Glioblastoma Multiforme	none	Surgery, radiotherapy, chemotherapy	Radioactive implant	Alive with disease, 12 mon (24 mon from orig Dx)
5	87	F	Lt parietal lobe	Malignant Astrocytoma	none	none	Radiotherapy and chemotherapy	Dead, 2 mon
6	61	F	Lt frontal lobe	Glioblastoma Multiforme	none	none	Radiotherapy	Alive with disease, 3 mon
7	36	M	Rt and Lt frontal lobe	Anaplastic Glioma	none	Biopsy and radiotherapy	Chemotherapy	Dead, 2 mon (18 mon from orig Dx)
8	40	M	Rt temporal lobe	Low Grade Astrocytoma	Neurofibromatosis	none	none	NED, 6 mon
9	32	M	Lt Temporal lobe	Glioblastoma Multiforme	none	Surgery, radiotherapy, chemotherapy	Chemotherapy and gene therapy	Dead, 8 mon (16 mon from orig dx)
10	10	M	Rt temporal lobe	Oligodendroglioma	Neurofibromatosis	none	none	NED, 2 mon
11	71	M	Rt temporal lobe	Glioblastoma Multiforme	none	none	Radiotherapy and chemotherapy	Alive with disease, 6 mon
12	58	F	Rt parietal lobe	Glioblastoma Multiforme	none	Biopsy and radiotherapy	Radiotherapy and chemotherapy	Alive with disease, 5 mon
13	89	M	Lt frontoparietal region	Glioblastoma Multiforme	none	none	Radiotherapy	Dead, 1 mon

[a]Dx = diagnosis, Tx = treatment, NED = no evidence of disease, Lt = left, Rt = right, mon = months.

TABLE 2
Control Patient Data[a]

Patient number	Age at Dx (yr)	Sex	Location of lesion	Diagnosis	Associated conditions	Tx before surgery	Tx after surgery	Status (mon after surgery)
1	24	F	Rt temporal lobe	Seizure disorder	none	none	none	No seizures, 6 mon
2	52	F	Rt temporal lobe	Seizure disorder	none	none	none	No seizures, 9 mon
3	36	M	Lt temporal lobe	Seizure disorder	none	none	none	Marked decrease in seizures, 7 mon

[a]Dx = diagnosis, Tx = treatment, Lt = left, Rt = right, mon = months.

RESULTS

The fatty acid composition, in terms of total lipid extract and phospholipid fraction, for glioma samples and control brain samples are shown in Table 3. Since no significant differences were observed in terms of lipid content between tumors arising from different regions of the brain, all the glioma data were combined for comparison with control brain tissue. The percentage of fatty acids present as PUFA was essentially identical in the normal brain and glioma samples; mean values were 26.9 ± 2.2% and 27.5 ± 3.1%, respectively. However, levels of the predominant n-3 PUFA, docosahexaenoic acid (DHA) (22:6n-3), were significantly reduced in the glioma samples ($P = 0.029$); mean values were 4.8 ± 2.9% compared with 9.2 ± 1.0% in the normal brain samples. A significant reduction in glioma DHA content was also observed in terms of the total phospholipids; mean values in the glioma samples were 4.6 ± 2.1% compared with 9.6 ± 0.8% in the normal brain samples ($P = 0.002$) (Fig. 1). Measurements of the fatty acid distribution of the four phospholipid fractions PE, PS, PC, and PI revealed further evidence of a reduction in the DHA content of the glioma tissue compared with the normal brain. The DHA content of the PE and PS fractions in the glioma samples was only some 50% of that observed in the samples taken from nonmalignant brain tissue; however, these reductions were not statistically significant (Fig. 1).

Differences were also noted in the n-6 PUFA content between glioma and normal brain samples. The glioma content of the n-6 PUFA LA (18:2n-6) was found to be significantly greater ($P < 0.05$) than that observed in the control samples in terms of total lipids (Table 3). A similar pattern was observed in terms of total phospholipids, but this was not statistically significant ($P = 0.073$).

These data were used to determine the unsaturation index (UI) of the total lipid and phospholipid fractions (13). Table 4 shows the mean UI of glioma and control tissue for the total lipid, total phospholipid, and for each of the phospholipid fractions. Although none of the differences reached statistical significance, there was a consistent trend, with the glioma UI being lower than that observed in the nonmalignant control brain samples for all lipid fractions.

In four samples, three tumor and one control, tissue samples were obtained from two different areas, to determine possible differences in fatty acid content due to sample heterogeneity. In all four specimens, no significant alteration in fatty acid distribution was observed due to variation in the area of tissue sample analyzed.

TABLE 3
Total Lipid and Phospholipid Fatty Acid Levels (mole %) in Tumor and Control Samples[a]

	Total lipid[b] (%)		Total phospholipid[b] (%)	
Fatty acid	Tumor ($n = 13$)	Control ($n = 3$)	Tumor ($n = 13$)	Control ($n = 3$)
16:0	26.3 ± 4.9	24.2 ± 1.8	28.3 ± 4.5	23.8 ± 1.9
18:0	14.7 ± 4.2	22.4 ± 0.6[c]	15.6 ± 3.4	22.7 ± 0.7[c]
16:1n-7	4.0 ± 1.6	1.3 ± 0.5[c]	3.4 ± 1.1	1.2 ± 0.5[c]
18:1n-9	24.0 ± 4.2	22.7 ± 3.4	23.7 ± 3.0	22.8 ± 3.7
20:1n-9	0.7 ± 0.5	1.3 ± 0.5	0.9 ± 0.5	1.3 ± 0.5
18:2n-6	4.3 ± 2.3	0.9 ± 0.0[c]	3.4 ± 2.1	0.8 ± 0.0[d]
20:3n-6	2.6 ± 1.7	1.0 ± 0.1	1.9 ± 0.9	0.9 ± 0.1
20:4n-6	9.0 ± 3.2	9.8 ± 1.5	10.8 ± 2.3	9.5 ± 1.3
22:4n-6	4.4 ± 3.0	5.4 ± 0.3	3.9 ± 1.4	5.6 ± 0.3
20:5n-3	0.2 ± 0.3	0.1 ± 0.1	0.1 ± 0.1	ND[e]
22:5n-3	0.5 ± 0.4	0.4 ± 0.1	1.5 ± 2.2	0.4 ± 0.1
22:6n-3	4.8 ± 2.9	9.2 ± 1.0[c]	4.6 ± 2.1	9.6 ± 0.8[c]

[a]Each value is the mean ± SD. [b]The values do not add up to 100% because only the most prevalent fatty acids are listed. [c]$P < 0.05$. [d]$P = 0.073$. [e]ND = not detectable.

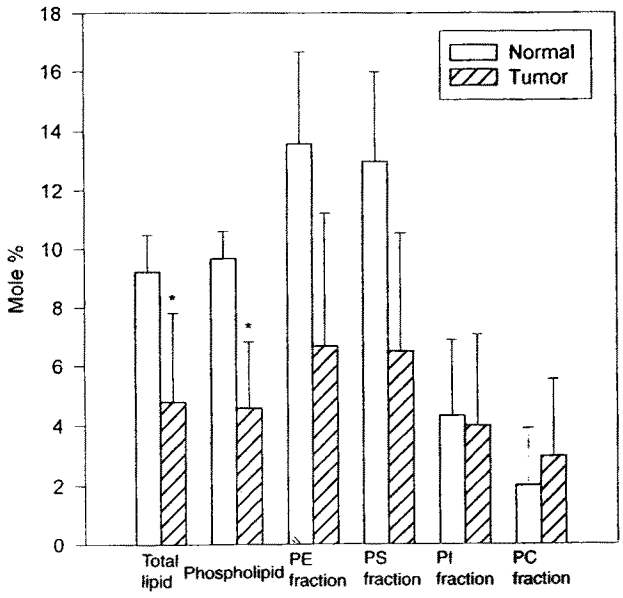


FIG. 1. Docosahexaenoic acid (DHA; 22:6n-3) levels in control and tumor brain tissue samples for the measured lipid fractions. Error bars show ± SD. PE, phosphatidylethanolamine; PS, phosphatidylserine, PI, phosphatidylinositol; PC, phosphatidylcholine. *$P < 0.03$.

TABLE 4
Unsaturation Index: Tumor vs. Control[a]

	Total lipid	Total phospholipid	PE	PS	PC	PI
Tumor	136.6 ± 13.9	135.2 ± 19.7	154.5 ± 47.4	88.9 ± 12.7	65.6 ± 33.6	90.6 ± 20.0
Control	148.8 ± 8.7	150.9 ± 4.0	198.4 ± 20.6	130.0 ± 17.4	77.5 ± 19.4	115.2 ± 15.1

[a]Unsaturation index is the average number of double bonds, on a molar basis, in the fatty acid mixture × 100. Values are mean ± SD. All differences between tumor and control with $P > 0.05$. PE = phosphatidylethanolamine, PS = phosphatidylserine, PI = phosphatidylinositol, and PC = phosphatidylcholine.

DISCUSSION

The current findings indicate that the percentage of total fatty acids present as PUFA in samples of malignant glioma tissue was essentially identical to that determined in normal brain tissue. However, there were marked differences between malignant and normal brain tissue in terms of the content of individual PUFA. A significant reduction in both total lipid and total phospholipid was noted in levels of the n-3 PUFA, DHA, in the glioma cells compared with normal brain tissue. DHA is the major n-3 PUFA in the CNS, being found in unusually high concentrations in synaptic membranes (14), the retina (15), and in particular the brain (16). The precise role that DHA plays in the CNS remains ill-defined. However, its importance is readily evident from observations indicating that dietary deprivation of n-3 PUFA is correlated with impairment of visual acuity (17) and learning ability (18). This might reflect, in part, alterations in membrane fluidity and functional properties of membrane proteins (6). In contrast, levels of the n-6 PUFA, linoleic acid (LA), were higher in the glioma tissue samples compared with control tissue. Thus, malignant human glioma tissue does appear to have an altered PUFA composition compared to nonmalignant brain tissue. These changes resulted in an apparent decrease in the UI of the glioma tissue, although the extent of this reduction did not reach acceptable levels of significance.

These data are in close agreement with previously published, even though limited, data on the fatty acid content of normal and malignant brain tissue. Stein *et al.* (19) analyzed the fatty acid composition of ten brain tumors, consisting of nine primary and one metastatic bronchogenic cancer. Due to the limited technology available at that time, only results for fatty acids up to 18 carbons in length were reported. These revealed that all glioma tissue analyzed contained increased levels of LA when compared with normal overlying cortical tissue removed from the same patient. An increase in LA content of neoplastic human tissue was also noted by White (13) from an analysis of 15 intracranial tumors, of which 7 were gliomas. White further reported decreased amounts of DHA in the PE and PC phospholipid fractions in these intracranial tumors compared with normal brain tissue. These data are consistent with the findings presented here. The mean glioma DHA content reported for the PE fraction was 6.8% compared with a value of 6.7 ± 3.7% from the current study; PC DHA content was also very similar between the two studies. Similar alterations in LA and DHA PE phospholipid fractions were observed by Sun and Leung (20). More recently, Ledwozyw and Lutnicki (21) published results for 8 gliomas and 10 meningiomas. The DHA composition for the glioma samples was again similar to the results presented here, with a mean total lipid DHA content of 5.1 ± 0.5% compared with our value of 4.8 ± 2.9% obtained in the current study.

The control tissue was obtained from three individuals who all exhibited seizure disorders. It should be noted that free fatty acid accumulation in the whole brain is a primary event following drug-induced seizures (22). Arachidonic acid appears to be the major fatty acid accumulated during these seizures, although increases in DHA have been noted experimentally (23). Thus, there exists the possibility that the values determined in the control patients in the current study might not be a true reflection of "normal" brain tissue. However, the fatty acid distribution data for the control tissue presented here are consistent with previously published data, and thus do appear to be representative of nonmalignant brain tissue (13,24,25).

The current observations of an increase in the content of LA and a decrease in 6-desaturated PUFA such as DHA further support the hypothesis that malignant cells exhibit an altered PUFA composition. Cheeseman *et al.* (4) reported a significant decrease in the PUFA content, in particular arachidonic acid (20:4n-6) and DHA, of hepatoma cells compared with normal rat hepatocytes. In contrast, the LA content of the malignant cells was elevated. These findings have been confirmed in other hepatoma cell lines (5,26). These alterations in malignant cell PUFA composition led to a reduced UI value for the malignant cells compared with that determined in the normal hepatocytes (26), an observation confirmed in the current study. A similar alteration in malignant cell PUFA content has been observed *in vivo*. Chaudry *et al.* (7) noted that malignant prostatic tissue had reduced levels of arachidonic acid (20:4n-6) and docosapentaenoic acid (22:5n-3) compared with benign prostatic tissue. This might reflect a lack of, or reduced activity of, the enzyme Δ^6 desaturase, required to desaturate LA and ALA to their respective products in malignant cells. Human cell lines exposed to oncogenic viruses, radiation, and chemical carcinogens show reduced rates of Δ^6-desaturation (27). Moreover, human lung cancer tissue transplanted into nude mice shows an absence of Δ^6-desaturase activity (28). Whatever the precise mechanism involved, it is important to note that as a consequence of this altered PUFA composition and reduction in UI values, lipid peroxidation should be significantly decreased in tumor cells

and tissues compared with the corresponding normal tissue (4). Many anticancer therapies, including irradiation, chemotherapy, hyperthermia, and photodynamic therapy, kill tumor cells by processes involving free radical generation leading to lipid peroxidation. Hence, malignant cell resistance to such therapy might be due, in part, to the altered PUFA composition, leading to a lower UI, and resultant decreased lipid peroxidation.

As noted earlier, tissue PUFA content will depend to a large extent on the diet; we are what we eat. The same principle applies to malignant cells, which derive all their PUFA from the host. Dietary studies demonstrate clearly that feeding tumor-bearing animals diets supplemented with particular fatty acids results in specific alterations in tumor cell fatty acid composition (29,30). Similarly, incubating malignant cells in culture with varying levels of different PUFA leads to specific, PUFA-dependent, changes in malignant cell PUFA content (31). The rate-limiting Δ^6 desaturase step can be bypassed by directly supplying preformed PUFA that contain 6-desaturation, such as gamma-linolenic acid (GLA, 18:3n-6). Such alterations in PUFA content and composition are associated with selective malignant cell kill; normal cells appear relatively unaffected (32). The mechanism for this cytotoxic effect appears to involve free radical generation and lipid peroxidation; it can be blocked by the addition of antioxidants (33). In addition, PUFA-enriched tumor cells exhibit increased susceptibility to subsequent treatments involving an oxidative stress, such as Adriamycin, photodynamic therapy, and iron loading (31,34).

Dietary supplementation with PUFA has been demonstrated to alter the PUFA composition of not only normal brain tissue (35,36) but also that of intracerebrally implanted tumor tissue (37). Indeed, intravenously administered fatty acids were more rapidly incorporated into the implanted tumor cells than the normal brain tissue. This ability to alter glioma cell PUFA composition might lead to an enhancement in the susceptibility of glioma cells to anticancer therapies which involve an oxidative stress, such as radiation and particular chemotherapeutic agents. These changes in PUFA composition are not tumor-specific, raising the concern that this approach might also increase the risk of normal tissue morbidity to these anticancer therapies. However, *in vitro* data suggest that normal cells are relatively unaffected by PUFA enrichment (32,38). Moreover, preliminary data from *in vivo* studies in the pig indicate that the administration of dietary PUFA for up to 16 wk after irradiation significantly reduced the severity of radiation-induced injury to the spinal cord (39).

In summary, the results of the present study indicate that the DHA content of glioma tissue is significantly reduced compared with normal brain tissue. In contrast, LA content is increased in the glioma cells. The net result of this altered PUFA content is likely to be a reduction in the susceptibility of these glioma cells to anticancer therapies which utilize free radical generation and lipid peroxidation pathways to cause tumor cell kill. We hypothesize that manipulating glioma PUFA content by dietary PUFA supplementation offers the promise of selectively increasing the susceptibility of the tumor cells to anticancer therapy. Indeed, intracerebral injections of GLA in glioma patients for 10 d (1 mg/day) after subtotal excision of the tumor, followed by radiotherapy, led to tumor regression and increased patient survival with no evidence of normal tissue toxicity (40). Thus, the addition of PUFA to the clinical armamentarium might lead to an improved therapeutic outcome for what remains an aggressive and often fatal neoplasm.

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Increasing Dietary Linoleic Acid in Young Rats Increases and Then Decreases Docosahexaenoic Acid in Retina But Not in Brain

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ABSTRACT: The accumulation of fatty acids in retina, brain, liver, and plasma of 30-day-old rat pups consuming various levels of linoleic acid (LA, 18:2n-6) and constant α-linolenic acid (ALA, 18:3n-3) is reported. Dams were fed graded levels of LA during gestation and lactation, and the pups were maintained on the diet of their dams until the end of the brain growth spurt at 30 d of life. Milk, and pup brain, retina, liver, and plasma were analyzed quantitatively for fatty acid profile. The percentage of docosahexaenoic acid (DHA, 22:6n-3) in retina increased from an LA-deficient dietary level, peaked at the 9:1 (LA/ALA) level, then fell for the 41:1 and 69:1 levels. In contrast, the brain DHA percentage was unaffected by dietary LA levels. Retinal unsaturated fatty acid levels paralleled liver and plasma levels. The milk fatty acid composition mirrored the diets. These data show that the retinal fatty acid composition responds sensitively to dietary fatty acid composition, similar to liver and plasma, while the brain unsaturate composition is nearly independent of dietary composition.

Lipids 31, 1289–1298 (1996).

Polyunsaturated fatty acids (PUFA) are the major components in the membrane structural lipids of neural tissues, particularly the brain and retina, which are rich in docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6) (1,2). Most PUFA are deposited in brain and retina during the brain growth spurt, when brain growth exceeds that of other organs (3–5). It is during this period that the dietary demand is highest for essential brain components. Several studies indicate that nutritional deficiency during the brain growth spurt results in lasting impairment of function which is not fully corrected during subsequent periods of repletion (3,6).

The fatty acid (FA) composition of most tissues is related to dietary supply in early development (7–9), although the FA composition of the brain is remarkably constant among free-living species (10). Experimentally, it is difficult but possible to alter the brain FA content. For instance, rats fed a sunflower oil diet with 70% linoleic acid (LA, 18:2n-6) content and 0.1% α-linolenic acid (ALA, 18:3n-3) through four generations exhibited low levels of DHA, and high levels of docosapentaenoic acid (DPA, 22:5n-6) in brain cells and nervous tissues compared with those fed soy oil diet with a more balanced precursor FA concentration (55% LA and 6.7% ALA) (11). Very low dietary levels of the C_{18} parent FA during the brain growth period result in decreased levels of the long-chain PUFA (LC-PUFA), like AA or DHA, which can affect normal visual function and learning ability (12–14).

It has been known for decades that the predominant dietary C_{18} PUFA serve as precursors for desaturation and elongation to LC-PUFA, which are required as substrates for a variety of potent bioactive compounds including the prostaglandins, thromboxanes, and leukotrienes. In addition, LA and ALA are competitive substrates for desaturation and elongation (15–17), and their relative proportion in the diet affects levels of LC-PUFA found in various tissues. For instance, weanling (18) or adult rats (19) fed increasing dietary ratios of LA to ALA show higher tissue levels of DPA and lower levels of DHA. In pregnancy, higher ratios of n-3 to n-6 FA in the maternal diet result in higher concentration of n-3 FA and lower n-6 FA in pup tissues (20,21).

Although the brain and retina are similar tissues close in proximity, nutrient transport from the blood is governed by separate structures. The blood–brain barrier (BBB) helps regulate PUFA uptake and metabolism from the plasma into the brain (22,23). Some inferential information on *in vivo* FA transport across the BBB is available (24,25), although many details remain obscure, while FA transport across the blood–retina barrier (BRB) is poorly understood.

The goal of this study was to isolate the effects of dietary n-6/n-3 ratio from that of varying absolute dietary levels on brain, retina, and liver accumulation of n-3 FA in young rat pups under conditions where ALA is constant and marginally meets dietary requirements (2 g/kg diet) (12), and LA is at deficient (1.4 g/kg diet), normal (16.6 g/kg diet), or very high levels (90, 144 g/kg diet). We hypothesize that in brain, retina,

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Abbreviations: AA, arachidonic acid (20:4n-6); ALA, α-linolenic acid (18:3n-3); ALAD, α-linolenic acid (18:3n-3) deficient; ANOVA, analysis of variance; BBB, blood–brain barrier; BF_3, boron trifluoride; BRB, blood–retina barrier; DHA, docosahexaenoic acid (22:6n-3); DPA, docosapentaenoic acid (22:5n-6); EFA, essential fatty acid; EPA, eicosapentaenoic acid (20:5n-3); FA, fatty acid; FAME, fatty acid methyl esters; GC, gas chromatography; LA, linoleic acid (18:2n-6); LC-PUFA, long-chain polyunsaturated fatty acids; PUFA, polyunsaturated fatty acids; TBHQ, *t*-butylhydroquinone; T/T, triene/tetraene, Mead acid (20:3n-9)/arachidonic acid (20:4n-6); UI, unsaturation index.

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and liver the accumulation of n-3 series FA is attenuated at high dietary LA/ALA ratio even though the dietary ALA is low but sufficient to meet dietary requirements. We also report the relationship between the dietary fat compositions and n-3 and n-6 deposition in milk and pup tissue.

MATERIALS AND METHODS

Animals. Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Wilmington, MA) and were housed individually in stainless-steel mesh cages in a room where temperature and humidity were maintained at 23 ± 1°C and 60–70%, respectively, with light from 0800 to 2000. Animals had free access to tap water and food. After the rats were acclimated to the environment, five-week-old female rats were fed an ALA-deficient diet (ALAD) with adequate LA levels for 4 wk to deplete body stores of excess ALA while maintaining reproductive capacity (26,27). The gross composition of this lead-in diet and its FA composition are shown in Tables 1 and 2. Dams were divided into four groups (n = 4/group) assigned to match average body weight in each group but otherwise at random. The nine-week-old dams were switched to experimental diets and bred with chow-fed nine-week-old male rats after checking vaginal smear cell patterns. Conception was confirmed by the presence of vaginal plugs and a smear of vaginal cells to detect the presence of spermatozoa. Pups were weaned to the diet of their dam and maintained on the diets until they were sacrificed at 30 days old.

Diets. The experimental diets were modified from the AIN-76A formulation containing 20% of oil by weight, and made up commercially (Dyets, Inc., Bethlehem, PA). The diet maintained the same nutrition density as AIN-76A. Composition of diets is shown in Table 1. α-Tocopherol (0.2 g/kg; 500 IU/gm) and *t*-butylhydroquinone (TBHQ, 0.02%) were added to oils as antioxidants. The oil blend, energy components, and FA compositions of the four experimental diets are given in Table 2. Safflower oil, a source of LA, linseed oil, a source of ALA, and hydrogenated coconut oil were blended to achieve the desired FA composition. The ALA content of the oils was constant in the four diets while the LA level was varied in order to obtain LA/ALA ratios of 0.6, 9, 41, and 69. The ALA level was about 2 g/kg diet (0.4% of energy) and was sufficient to meet the dietary requirements for rats (12). The dietary LA concentration of groups 0.6, 9, 41, and 69 were 1.4, 16.6, 90, and 144 g/kg diet, respectively. The 0.6 diet is LA-deficient since the LA content is much less than the minimal LA dietary requirement in rats (28). The 9, 41, and 69 diets are referred to as the "replete" diets since they meet requirements for both LA and ALA. A fifth diet was prepared with very low levels of ALA designed as an ALAD diet. It contained sufficient LA (2.6% of energy) to satisfy the minimal requirement in rats (28).

TABLE 1
Composition of Diet Fed to the Different Experimental Groups

Ingredient	g/kg diet
Casein	235
Sucrose	271
Oil[a]	200
DL-methionine	3.5
Cornstarch	176
Cellulose	59
Vitamin E acetate (500 IU/g)	0.2
Salt mix[b]	41
Vitamin mix[c]	12
Choline bitartrate	2.4

[a]Oil was supplied from linseed, safflower, and hydrogenated coconut oil (Dyets Inc., Bethlehem, PA).

[b]Salt mix (mg/kg diet) included calcium phosphate dibasic, 20,600; sodium chloride, 3,050; potassium citrate monohydrate, 9,060; potassium sulfate, 2,140; magnesium oxide, 989; manganous carbonate, 144; ferric citrate, 247; zinc carbonate, 66; cupric carbonate, 12; potassium iodate, 0.412; sodium selenite, 0.412; chromium potassium sulfate 12 H_2O, 22.7 (AIN-76A salt mix DYETS #200000, Dyets Inc.).

[c]Vitamin mix (mg/kg diet) included Thiamin HCl, 7.08; riboflavin, 7.08; pyridoxine HCl, 8.26; niacin, 35.4; calcium pantothenate, 18.9; folic acid, 2.36; biotin, 0.24; vitamin B12, 11.8; vitamin A palminate (500,000 U/g), 9.44; vitamin D3 (400,000 U/g), 2.95; vitamin E acetate 118; and menadione sodium bisulfite, 0.944 (AIN-76A vitamin mixture, DYETS #300050; Dyets Inc.).

Sample collection. At days 10, 15, and 20 post-parturition, dams were anesthetized with ether, and milk was collected after injection of oxytocin. The 30-day-old pups were weighed, anesthetized with CO_2, and decapitated. Blood was collected into a small quantity of iced saline with EDTA as anticoagulant, and plasma separated immediately by centrifugation. The liver and brain were removed quickly, rinsed with iced saline, weighed and wrapped in foil and frozen in liquid nitrogen. Retinas were removed and placed in iced saline before being frozen. All samples were stored under N_2 and kept in a –80°C freezer until analysis.

Lipid analysis. Six pups were randomly chosen from each group for plasma, liver, and brain lipid analysis. For retinal analysis, 2 or 3 pup retinas (4–6 retinas) were pooled as one sample with a total of six samples analyzed for each group to determine the retinal FA composition. Milk and pup tissue lipids were extracted, saponified, and methylated. Briefly, total lipids were extracted from aliquots of tissue homogenate according to the method of Bligh and Dyer (29). The extracted lipids were saponified by adding 0.5N NaOH in 95% methanol solution and heating at 60°C for 10 min. Methanolic boron trifluoride (BF_3, 14%) was added, heated at 100°C for 2 min to yield FA methyl esters (FAME). FAME were removed by adding hexane and saturated NaCl. The purified FAME were taken up in hexane, with methyl heptadecanoate (Me17:0; 99+% pure; Sigma Chemicals, St. Louis, MO) added quantitatively as an internal standard. The mixture was stored under N_2 and kept in a –80°C freezer until analysis. A Carlo Erba (Milano, Italy) 5160 Mega series gas chromatograph (GC) with a J&W (Folsom, CA) DB23 fused silica capillary column (30 m, 0.32 mm i.d., 0.25 μm film) with H_2 as carrier gas was used for FAME samples. The oven was set to ramp from 60 to 255°C with three plateaus over 40 min. The individual FA peaks were identified by comparison of retention times with a standard mixture ("68A", Nu-Chek-Prep,

TABLE 2
Oil Composition, EFA Composition, Energy Composition, and Fatty Acid Composition of the Different Experimental Diets

	Diet groups				
	0.6[a]	9[b]	41[c]	69[d]	ALAD[e]
Oil composition (g/kg diet)					
Linseed oil	4	4	4	4	0
Safflower oil	0	25	105	196	21
Hydrogenated coconut oil	196	171	91	0	179
EFA composition (g/kg diet)					
Linoleic acid	1.4	16.6	90	144	13.2
α-Linolenic acid	2.2	1.8	2.2	2.1	0
Energy composition (% of energy)					
Oil	40	40	40	40	40
Linoleic acid	0.3	3.3	18	29	2.6
α-Linolenic acid	0.4	0.4	0.4	0.4	0
Fatty acid composition (%)					
10:0	5.0	1.3	0.4	0.0	2.9
12:0	30.4	33.2	17.6	1.1	31.2
14:0	36.4	30.9	11.9	1.4	30.5
16:0	13.6	16.3	11.1	10.4	19.5
18:0	11.3	7.4	5.6	2.4	7.5
18:1n-9	1.3	1.8	7.2	11.5	1.7
18:2n-6	0.7	8.3	45.0	72.2	6.6
18:3n-3	1.09	0.92	1.10	1.05	nd[f]
18:2n-6/18:3n-3 = LA/ALA	0.6	9	41	69	nc[f]

[a]0.6 group = the ratio of linoleic acid (LA) to α-linolenic acid (ALA) was 0.6 in the diet = LA deficient diet. EFA, essential fatty acid.
[b]9 group = the ratio of LA to ALA was 9 in the diet.
[c]41 group = the ratio of LA to ALA was 41 in the diet.
[d]69 group = the ratio of LA to ALA was 69 in the diet.
[e]ALAD = α-linolenic acid (18:3n-3, ALA) deficient diet.
[f]nd = Not detected; nc = not calculated.

Elysian, MN; "PUFA2", with added 20:3n-9, Matreya, Pleasant Gap, PA). Acceptable chromatographic separation of all FA was verified for all samples. Response factors were derived from the standard mixture and used to calculate relative concentrations. The FA compositions are expressed as weight percentage of total FA from carbon 8 to carbon 22.

Statistical methods. All data are presented as the mean ± standard deviation. Statistical differences among the four groups were determined using analysis of variance (ANOVA). When the *P*-value was significant at the 0.05 level, Tukey's *t*-test was applied pairwise to detect group differences. All statistical analyses were performed using MINITAB for Windows, version 10 (Minitab Inc., State College, PA).

RESULTS

Organ weights. Neither liver nor brain weights were significantly different among the groups, indicating that liver and brain growth in rats are not impaired by the high ratio of LA/ALA. The range of brain and liver weights was 1.40–1.43 g and 3.0–3.7 g, respectively. No gross differences in fertility were observed among dietary groups.

Milk. The FA composition of rat milk at 10, 15, and 20 d postpartum was not significantly different within dietary treatments. We therefore present only the composition of the milk at 10 d postpartum, shown in Table 3. The major n-6 and n-3 FA were LA and ALA, respectively, and were closely related to the maternal diet. The range of ALA was from 0.7 to 1.0% and was not significantly different among the groups, which was consistent with the equivalent dietary content of ALA from 0.9 to 1.1. In contrast, the LA content increased in direct proportion to their abundance in the diet, where LA was 2, 13, 32, and 50%, and the sum of n-6 series FA was 3, 16, 37, and 54% for groups 0.6, 9, 41, and 69, respectively.

Interestingly, there was no significant change in AA among the replete groups, but AA was significantly lower in the 0.6 group. Although the milk ALA concentration was independent of the LA concentration, the desaturation/elongation product of ALA, eicosapentaenoic acid (EPA, 20:5n-3) was observed only in the groups 0.6 and 9 and was below detectable limits in the others, suggesting that the higher LA levels inhibited conversion. DHA levels were significantly lower in the 69 group compared to the 0.6, 9, and 41 groups, although no statistically significant differences were found among the latter three. Taken together, the four groups show a very clear trend as mean DHA percentages drop from 0.37% to trace levels in 0.6, 9, 41, and 69 groups, respectively. This decreasing maternal DHA supply, on a relative basis, may in part be responsible for trends seen in pup tissue DHA percentages.

TABLE 3
Milk Fatty Acid Composition from Rats Fed the Different Experimental Diets on Day 10 Postpartum

	Groups			
	0.6	9	41	69
Fatty acids				
Sum sat'd[a]	71.4 ± 6.86[b]	60.7 ± 3.13	46.7 ± 4.64	30.8 ± 1.02
Sum mono[c]	23.7 ± 3.71	20.6 ± 2.11	13.5 ± 0.62	12.6 ± 2.83
20:3n-9	0.14 ± 0.24	0.79 ± 0.15	1.71 ± 0.18	1.56 ± 0.30
18:2n-6	2.17 ± 1.53[a]	12.6 ± 1.03[b]	31.5 ± 2.16[c]	49.6 ± 5.50[d]
18:3n-6	0.03 ± 0.05[a]	0.28 ± 0.03[a]	0.48 ± 0.19[a]	0.51 ± 0.47[a]
20:3n-6	0.09 ± 0.16[a]	0.81 ± 0.10[ab]	1.20 ± 0.20[b]	1.18 ± 0.28[b]
20:4n-6	0.54 ± 0.44[a]	1.77 ± 0.26[b]	2.63 ± 1.06[b]	2.70 ± 0.82[b]
22:4n-6	trace[d]	0.56 ± 0.11[a]	1.00 ± 0.50[a]	0.30 ± 0.52[a]
22:5n-6	trace	trace	0.09 ± 0.15	trace
Sum n-6[e]	2.83 ± 2.17[a]	16.0 ± 1.33[b]	36.9 ± 3.90[c]	54.3 ± 3.57[d]
18:3n-3	0.78 ± 0.30[a]	0.84 ± 0.06[a]	1.01 ± 0.05[a]	0.73 ± 0.08[a]
20:5n-3	0.47 ± 0.21[a]	0.35 ± 0.06[a]	trace	nd[d]
22:5n-3	0.38 ± 0.33[a]	0.43 ± 0.39[a]	0.24 ± 0.24[a]	nd
22:6n-3	0.37 ± 0.16[a]	0.27 ± 0.05[a]	0.17 ± 0.18[a]	trace
Sum n-3[f]	2.00 ± 0.84[a]	1.89 ± 0.38[a]	1.41 ± 0.39[a]	0.73 ± 0.08[b]
PUFA[g]	4.83 ± 3.00[a]	17.9 ± 0.98[b]	38.3 ± 4.27[c]	55.1 ± 3.65[d]
UI[h]	39.8 ± 13.5[a]	68.7 ± 3.83[b]	107 ± 13.9[c]	136 ± 2.34[d]
n-6/n-3[i]	1.41 ± 0.47	8.46 ± 2.43	26.1 ± 5.62	74.1 ± 2.89
LA/ALA[j]	2.78 ± 0.83	15.0 ± 2.09	31.2 ± 3.53	67.7 ± 1.93
T/T[k]	0.26 ± 0.24	0.45 ± 0.11	0.65 ± 0.34	0.58 ± 0.06

[a]Sum of 8:0, 10:0, 12:0, 14:0, 16:0, and 18:0; sat'd saturated.
[b]Values are percentage of total fatty acids and expressed as mean ± SD; Values with different superscript letters in the same row are significantly different ($P < 0.05$) from any others by using the Tukey's *t*-test.
[c]Sum of 16:1n-7, 18:1n-9, and 18:1n-7; mono, monounsaturated.
[d]Trace amount less than 0.1% of total fatty acids; nd = not detected; nc = not calculated.
[e]Sum of n-6 series fatty acids including 18:2n-6, 18:3n-6, 20:3n-6, 20:4n-6, 22:4n-6, and 22:5n-6.
[f]Sum of n-3 series fatty acids including 18:3n-3, 20:5n-3, 22:5n-3, and 22:6n-3.
[g]Sum of n-6 and n-3 series of fatty acids; PUFA, polyunsaturated fatty acids.
[h]UI = unsaturation index = sum of the number of double bond · the percentage of fatty acids.
[i]n-6/n-3 = the ratio of sum of n-6 series fatty acids to sum of n-3 series fatty acids.
[j]LA/ALA = 18:2n-6/18:3n-3 = the ratio of linoleic (18:2n-6) to α-linolenic acid (18:3n-3).
[k]Triene/tetraene (T/T) = essential fatty acids deficiency index = the ratio of mead acid (20:3n-9) to arachidonic acid (20:4n-6).

Liver. Table 4 shows the FA composition of liver from 30-day old rats. The major n-6 FA were LA and AA, while DHA was the primary n-3 FA. The accumulation of PUFA in rat liver was about 52–55% and was not different among the replete groups. As shown in Figure 1, the AA percentage did not change among the replete groups, while DHA dropped in the presence of higher LA/ALA as it was observed to decrease from 15, 12, 6, to 4% among groups 0.6, 9, 41, and 69, respectively. This observation supports the hypothesis that n-6 FA compete for desaturation/elongation with n-3 FA.

The LA level, sum of n-6 or n-3 FA was unchanged between groups 40 and 70. However, a reciprocal accumulation of n-6 and n-3 FA was observed wherein the total n-6 FA increased from 9, 35, to 46% while the n-3 FA decreased from 27, 18, to 8% among groups 0.6, 9, and 41. The 0.6 group contained the greatest percentage of n-3 FA and the lowest percentage of n-6 series of FA. This observation again supports the interaction of dietary LA and ALA levels on the composition of liver FA.

The 0.6 group had significantly lower total PUFA levels than other groups, perhaps due to FA challenge by the inadequate levels of LA in diets. There was no significant difference in the unsaturation index (UI) among the four groups. The triene/tetraene (T/T) value, an essential FA deficiency index, was greater than 0.4 in the 0.6 group, indicating EFA deficiency. It is interesting to note that T/T reflected EFA deficiency under this relatively minor dietary deficiency, even though ALA was present at adequate dietary levels.

Plasma. FA composition of plasma from 30-day-old pups is shown in Table 5. LA and AA were the main n-6 plasma FA as was observed for liver, while DHA was the dominant n-3 FA. There were no significant differences in DHA level or the total amount of n-3 FA among the replete groups, although the 0.6 group showed significantly greater amounts of DHA, EPA, and total n-3 FA. A reciprocal accumulation of n-3 and n-6 FA was observed, in which the total n-3 FA decreased from 19, 6, 5, and 3%, while the n-6 FA increased from 10, 39, 60 to 69% within groups 0.6, 9, 41, and 69, respectively. The PUFA value increased from 29, 45, 64 to 71% among groups 0.6, 9, 41, and 69, respectively.

TABLE 4
Liver Fatty Acid Composition of 30-Day-Old Pups Fed the Different Experimental Diets[a]

	Groups			
	0.6	9	41	69
Fatty acids				
Sum sat'd	41.1 ± 1.96	36.4 ± 2.70	39.9 ± 1.61	39.2 ± 2.46
Sum mono	19.0 ± 0.86	10.7 ± 1.45	4.78 ± 0.67	4.82 ± 0.71
20:3n-9	3.72 ± 0.81	0.60 ± 0.18	0.76 ± 0.09	1.25 ± 0.20
18:2n-6	3.74 ± 0.19^a	11.7 ± 0.76^b	17.6 ± 0.70^c	17.8 ± 0.79^c
18:3n-6	trace	0.30 ± 0.09^a	0.87 ± 0.14^b	0.75 ± 0.23^b
20:3n-6	0.63 ± 0.27^a	1.03 ± 0.17^a	0.46 ± 0.04^a	0.42 ± 0.04^a
20:4n-6	4.43 ± 0.20^a	19.0 ± 1.12^b	21.7 ± 1.05^b	21.5 ± 2.30^b
22:4n-6	trace	0.76 ± 0.07^a	1.53 ± 0.25^b	2.05 ± 0.43^b
22:5n-6	trace	2.00 ± 0.24^a	4.00 ± 0.54^{a,b}	4.70 ± 0.93^b
Sum n-6	8.81 ± 0.56^a	34.8 ± 1.47^b	46.1 ± 0.66^c	47.3 ± 2.02^c
18:3n-3	0.21 ± 0.06^a	0.22 ± 0.05^a	0.12 ± 0.11^a	0.08 ± 0.07^a
20:5n-3	8.30 ± 0.55^a	0.48 ± 0.12^b	nd	nd
22:5n-3	3.45 ± 0.44^a	4.36 ± 0.52^a	2.33 ± 0.40^b	3.85 ± 1.42^a
22:6n-3	15.4 ± 1.11^a	12.4 ± 0.41^b	5.98 ± 0.72^c	3.52 ± 2.25^d
Sum n-3	27.3 ± 1.79^a	17.5 ± 0.28^b	8.42 ± 0.94^c	7.44 ± 1.16^c
PUFA	36.1 ± 1.59^a	52.3 ± 1.51^b	54.5 ± 1.32^b	54.7 ± 2.97^b
UI	209 ± 10.6^a	228 ± 5.04^a	207 ± 7.75^a	206 ± 16.1^a
T/T	0.84 ± 0.21	0.03 ± 0.01	0.03 ± 0.00	0.06 ± 0.01

[a]Values are percentage of total fatty acids and expressed as mean ± SD; trace amount less than 0.1% of total fatty acids; nd = not detected. Values with different superscript letters in the same row are significantly different ($P < 0.05$) from any others by using the Tukey's *t*-test. See Table 3 for abbreviations.

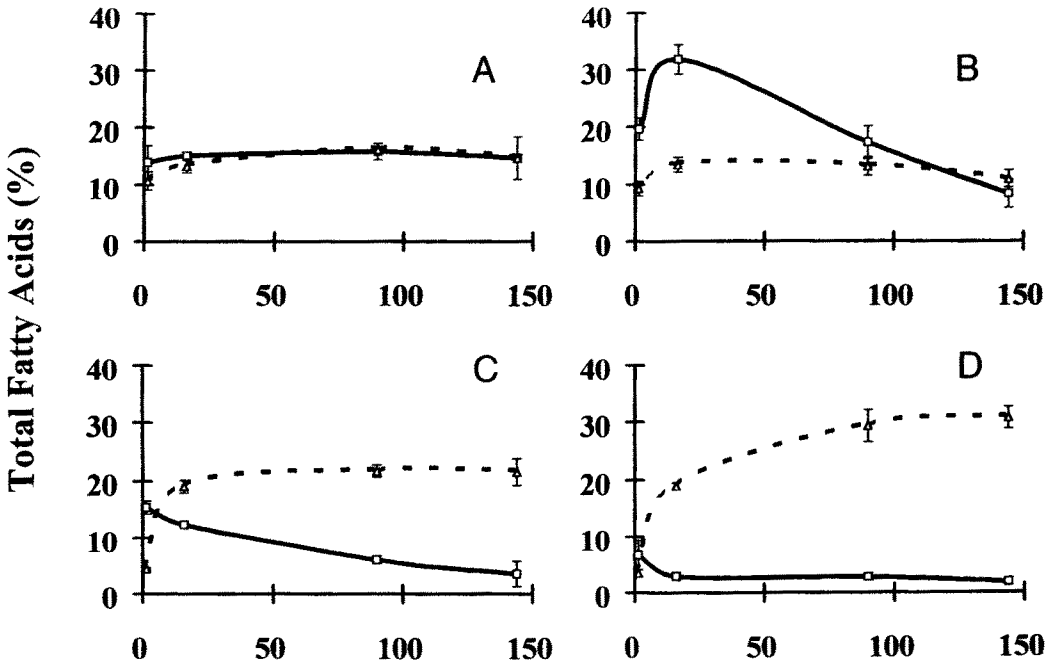


FIG. 1. Effect of increasing dietary linoleic acid (LA) concentrations with constant α-linolenic acid levels on docosahaxaenoic acid (DHA) (——) and arachidonic acid (- - - -) accretion in 30-day-old rat pups (mean ± SD) in (A) brain, (B) retina, (C) liver, and (D) plasma. The retinal DHA level responds sensitively to dietary LA as does liver and plasma, while the brain DHA level is unaffected.

TABLE 5
Plasma Fatty Acid Composition of 30-Day-Old Pups Fed the Different Experimental Diets[a]

	Groups			
	0.6	9	41	69
Fatty acids				
Sum sat'd	45.2 ± 6.02	45.8 ± 2.24	29.8 ± 3.45	20.8 ± 2.07
Sum mono	22.7 ± 1.69	9.32 ± 1.34	5.37 ± 0.68	7.14 ± 0.98
20:3n-9	3.21 ± 0.65	0.48 ± 0.13	0.43 ± 0.14	0.60 ± 0.14
18:2n-6	5.42 ± 0.46^a	17.5 ± 1.87^b	24.9 ± 1.50^c	32.9 ± 2.84^d
18:3n-6	0.25 ± 0.02^a	0.46 ± 0.08^a	0.90 ± 0.38^b	0.87 ± 0.21^b
20:3n-6	0.57 ± 0.18^a	0.69 ± 0.13^b	0.36 ± 0.09^c	0.29 ± 0.14^c
20:4n-6	3.45 ± 0.67^a	18.9 ± 0.60^b	29.3 ± 2.82^c	30.7 ± 1.89^c
22:4n-6	nd	0.52 ± 0.42^a	1.47 ± 0.46^b	1.45 ± 0.38^b
22:5n-6	0.07 ± 0.10^a	0.79 ± 0.46^a	2.89 ± 0.93^b	2.44 ± 0.74^b
Sum n-6	9.76 ± 1.29^a	38.9 ± 1.62^b	59.8 ± 2.59^c	68.6 ± 2.93^d
18:3n-3	1.11 ± 0.12^a	0.41 ± 0.10^b	0.31 ± 0.11^b	0.26 ± 0.06^b
20:5n-3	9.70 ± 1.82^a	0.79 ± 0.17^b	0.32 ± 0.16^b	0.09 ± 0.15^b
22:5n-3	1.66 ± 0.81^a	1.50 ± 0.74^a	1.24 ± 0.36^{a,b}	0.60 ± 0.28^b
22:6n-3	6.71 ± 2.66^a	2.90 ± 0.71^b	2.76 ± 0.64^b	1.89 ± 0.69^b
Sum n-3	19.2 ± 5.12^a	5.60 ± 1.63^b	4.63 ± 1.10^b	2.84 ± 1.02^b
PUFA	29.0 ± 6.32^a	44.5 ± 1.25^b	64.4 ± 3.20^c	71.4 ± 2.60^d
UI	160 ± 33.3^a	161 ± 9.79^a	223 ± 16.8^b	234 ± 10.2^b
T/T	0.93 ± 0.11	0.03 ± 0.01	0.01 ± 0.00	0.02 ± 0.00

[a]Values are percentage of total fatty acids and expressed as mean ± SD; trace amount less than 0.1% of total fatty acids; nd = not detected. Values with different superscript letters in the same row are significantly different ($P < 0.05$) by using the Tukey's *t*-test. See Table 3 for abbreviations.

TABLE 6
Brain Fatty Acid Composition from 30-Day-Old Pups Fed the Different Experimental Diets[a]

	Groups			
	0.6	9	41	69
Fatty acids				
Sum sat'd	46.7 ± 4.52	45.7 ± 3.25	36.4 ± 3.13	37.8 ± 3.65
Sum mono	21.7 ± 1.66	17.6 ± 1.76	16.3 ± 1.06	15.9 ± 2.30
20:3n-9	0.99 ± 0.26	0.24 ± 0.13	0.47 ± 0.10	0.56 ± 0.07
18:2n-6	0.66 ± 0.29^a	1.17 ± 0.14^{a,b}	1.55 ± 0.18^{b,c}	1.84 ± 0.37^c
18:3n-6	nd	nd	nd	nd
20:3n-6	1.00 ± 0.35^a	0.52 ± 0.09^b	0.58 ± 0.09^b	0.65 ± 0.06^b
20:4n-6	10.7 ± 1.60^a	13.3 ± 1.10^b	16.1 ± 0.53^c	14.6 ± 0.55^{b,c}
22:4n-6	1.85 ± 0.60^a	3.55 ± 0.55^b	5.24 ± 0.62^c	5.52 ± 1.07^c
22:5n-6	0.49 ± 0.45^a	2.19 ± 0.21^a	6.99 ± 1.51^b	7.71 ± 1.57^b
Sum n-6	14.7 ± 2.74^a	20.7 ± 1.48^b	30.4 ± 2.13^c	30.4 ± 1.99^c
18:3n-3	nd	trace	nd	nd
20:5n-3	0.43 ± 0.30^a	0.16 ± 0.13^b	0.09 ± 0.13^b	0.13 ± 0.18^{a,b}
22:5n-3	1.64 ± 0.80^a	0.60 ± 0.32^b	0.63 ± 0.13^b	0.62 ± 0.13b^b
22:6n-3	13.9 ± 2.94^a	15.0 ± 0.54^a	15.7 ± 1.43^a	14.6 ± 3.68^a
Sum n-3	15.9 ± 2.71^a	15.8 ± 0.81^a	16.4 ± 1.52^a	15.4 ± 3.83^a
PUFA	30.7 ± 4.76^a	36.5 ± 1.94^a	46.8 ± 3.32^b	45.7 ± 5.78^b
UI	175 ± 23.2^a	194 ± 10.1^a	240 ± 16.9^b	234 ± 30.6^b
T/T	0.09 ± 0.02	0.02 ± 0.01	0.03 ± 0.01	0.04 ± 0.00

[a]Values are percentage of total fatty acids and expressed as mean ± SD; trace amount less than 0.1% of total fatty acids; nd = not detected. Values with different superscript letters in the same row are significantly different ($P < 0.05$) by using the Tukey's *t*-test. See Table 3 for abbreviations.

Brain. The brain FA composition is presented in Table 6. The two major n-6 FA were AA and DPA, unlike liver and plasma where AA and LA predominate. The primary component of n-3 FA was DHA at 14–16% of total FA and was not affected by increasing dietary levels of LA as shown in Figure 1. There was no significant difference in the sum of n-3 FA. In this regard, the brain FA composition was more resistant to dietary influence than liver or plasma, as expected. The percentage of DPA, sum of n-6 FA, total PUFA, and UI were not significantly different between groups 41 and 69, but were lower in groups 0.6 and 9. No ALA was detected among the four groups. Here, the T/T value was slightly elevated in the 0.6 group, but was well below the 0.4 cutoff for EFA deficiency, suggesting that ALA masked the LA deficiency in the

TABLE 7
Retina Fatty Acid Composition of 30-Day-Old Pups Fed the Different Experimental Diets[a]

	Groups			
	0.6	9	41	69
Fatty acids				
Sum sat'd	47.6 ± 3.31	32.8 ± 5.30	39.1 ± 6.95	43.6 ± 8.65
Sum mono	16.5 ± 1.30	11.6 ± 1.73	17.0 ± 1.84	17.4 ± 2.47
20:3n-9	0.62 ± 0.09	0.27 ± 0.26	0.40 ± 0.21	0.77 ± 0.31
18:2n-6	1.21 ± 0.28^a	1.74 ± 0.37^a	3.42 ± 0.60^b	6.99 ± 1.94^c
18:3n-6	trace	0.01 ± 0.03^a	0.05 ± 0.08^a	0.16 ± 0.03^b
20:3n-6	0.32 ± 0.08^a	0.27 ± 0.27^a	0.26 ± 0.14^a	0.42 ± 0.12^a
20:4n-6	9.25 ± 1.47^a	13.4 ± 1.59^b	13.1 ± 1.24^b	10.9 ± 1.25^{a,b}
22:4n-6	1.44 ± 0.38^a	3.32 ± 0.95^{a,b}	3.26 ± 1.04^{a,b}	4.00 ± 1.90^b
22:5n-6	0.39 ± 0.13^a	2.85 ± 1.08^{a,b}	5.66 ± 1.29^b	6.93 ± 2.09^b
Sum n-6	12.6 ± 1.94^a	21.6 ± 3.37^b	25.7 ± 3.34^b	29.4 ± 5.10^b
18:3n-3	0.06 ± 0.09^a	0.04 ± 0.06^a	trace	0.10 ± 0.07^a
20:5n-3	trace	trace	trace	trace
22:5n-3	3.02 ± 1.61^a	1.89 ± 1.27^{a,b}	0.58 ± 0.49^b	0.60 ± 0.41^b
22:6n-3	19.6 ± 2.49^a	31.8 ± 2.88^b	17.2 ± 2.56^a	8.27 ± 1.91^c
Sum n-3	22.7 ± 2.51^a	33.8 ± 3.47^b	17.8 ± 2.91^c	8.97 ± 2.13^d
PUFA	35.3 ± 3.49^a	55.4 ± 6.51^b	43.5 ± 5.71^a	38.3 ± 6.73^a
UI	199 ± 17.2^a	298 ± 32.2^b	209 ± 29.2^a	182 ± 33.4^a
T/T	0.07 ± 0.02	0.02 ± 0.02	0.03 ± 0.02	0.07 ± 0.02

[a]Values are percentage of total fatty acids and expressed as mean ± SD; trace amount less than 0.1% of total fatty acids. Values with different superscript letters in the same row are significantly different ($P < 0.05$) by using the Tukey's *t*-test. See Table 3 for abbreviations.

brain. The AA level was significantly lower in the 0.6 group compared to the replete groups, and increased significantly from the 9 to the 41 group, while the 69 group was not significantly different from either.

Retina. Table 7 shows the FA composition of 30-day-old rat retina. In contrast to the brain, the retina n-3 FA composition responds sensitively to the dietary LA level. The percentage of n-3 FA was 34, 18, and 9% among groups 9, 41, and 69, respectively, corresponding to decreases in DHA from 32, 17, to 8% as shown in Figure 1. The major n-6 FA, AA, as well as DPA and the sum of n-6 FA, did not change in the replete groups but did drop significantly in the 0.6 group. That is, the increasing level of dietary LA did not affect the accumulation of n-6 FA in rat reina; however it decreased DHA accretion. High dietary levels of LA did not cause replacement of DHA by DPA as was observed in other studies (18,19). LA partially replaced DHA at high dietary levels, as the LA content was 1, 2, 3, and 7% among groups 0.6, 9, 41, and 69, respectively. Group 9 contained the highest amount of DHA, AA, PUFA, and UI compared with other groups and, as with the brain, the T/T value was less than 0.1 in all groups.

DISCUSSION

Bourre *et al.* (28) have investigated the effects of increasing dietary LA levels on FA accretion in retina, brain components, and other organs of 21-day-old rats, in a study similar to ours. They report constant AA levels in pup brain and retinas, while DHA dropped at high LA levels. The range of LA/ALA ratios investigated, from about 1 to 10, does not include the much higher levels attainable with common vegetable oils which are investigated here, and which showed substantial divergence between brain and retinal accumulation.

The highest dietary LA level reflecting a ratio of about 69:1 is close to the highest ratio attainable with commonly available oils while maintaining the overall calories as fat at 40%. At the other extreme of LA content, the 0.6 group was the lowest level practicably attainable while maintaining ALA at a constant, adequate level, and below requirements for LA (28). Plasma and liver of the 0.6 group (dietary LA level: 0.3% of energy) had a T/T ratio >0.4, indicating essential FA deficiency (30). The marginally adequate dietary ALA levels were apparently not sufficient to mask the T/T index at this LA intake.

As with all nutritional studies, experimental manipulation of macronutrient levels necessitates changes in absolute and relative amounts of other dietary constituents. Here, the changing LA dietary content was compensated using hydrogenated coconut oil, chosen because it contains no essential FA. Thus, the results may be influenced by the changing saturated FA levels because they are known to have an effect on, for instance, expression of the Δ9-desaturase. This study design permitted isolation of LA/ALA ratio while holding ALA constant. To the extent coconut oil FA effects can be discounted, results may therefore be ascribed to competition of LA with ALA.

Brain and retina DHA profiles differed markedly in their response to increasing dietary LA, as shown in Figure 1. High LA levels did not decrease accretion of brain DHA but had a strong effect on retinal DHA levels. In brain, there were no significant differences between DHA levels in any group, including the n-6 deficient group. Although recent

work suggests that dramatic differences in brain DHA content can be achieved with artificial rearing (31), our data is consistent with previous observations. In contrast, retinal DHA, and the sum of n-3 FA, increased from group 0.6 to group 9 and then decreased linearly in groups 41 and 69. This was the most dramatic difference in FA profiles detected in this study and indicates that the supply or metabolism of DHA differs substantially in these two presumably similar neural tissues.

Many studies show that the brain DHA concentration is resistant to dietary manipulation. Bourre *et al.* (32) pointed out that rat brain DHA was conserved even when dietary ALA is deficient for 7 mon. Arbuckle *et al.* (33) suggested that the quantity of dietary ALA rather than the dietary ratio of LA to ALA played a more important role in the accumulation of DHA in the brain. Anderson (34) demonstrated that the deposition of DHA in the brain was resistant to alterations at the early stage of brain growth spurt by feeding fish oil. Sheaff *et al.* (35) reported that conversion of DHA from ALA was not depressed by feeding high dietary LA level in young rats. Ponder *et al.* (36) showed that the DHA level in infant plasma or red blood cell was constant even in infants fed more ALA.

In contrast to brain, retina FA composition is manipulated by dietary LA/ALA level. The retina showed a maximal DHA level in group 9 and decreased significantly at higher ratios. Group 69 produced a very low retinal DHA content of 8%, which is comparable to the level found in retinal phosphatidylethanolamine of neonatal rhesus monkeys born to mothers fed n-3 deficient diets (37), but greater than the 2.5% of FA in guinea pig retinal phospholipids fed safflower oil through three generations (38). A recent study of formula-fed rat pups with LA/ALA from 0.08 to 10 maintained retinal DHA levels around 20% of total FA, suggesting that the more extreme ratios are required to produce measurable change (39). There is no data as to whether these changes are functionally significant.

In milk, ALA level was not affected by the LA level in the diets. The LA level in milk closely paralleled the level in the replete diets, at the level of about 31% according to linear regression. Since rat liver is an active desaturator, we conclude that the mammary gland takes up FA postprandially from the maternal circulation, probably as nonesterified FA after the action of lipoprotein lipase on circulating chylomicrons or chylomicron remnants. Lipoprotein lipase is active in the mammary gland and could explain the FA source (40), although it is not known if the mammary gland absorbs chylomicrons or remnants. The lower AA level found in the 0.6 group may be an indication of inadequate LA supply impairing function associated with uptake or secretion.

The n-3 FA composition of pup retina and liver was sensitive to the dietary LA level, while brain n-3 FA composition was not. Specifically, DHA accretion was much lower in pup liver and retina in the presence of high levels of dietary LA, while the brain DHA level was independent of LA levels. This result indicates that the retinal supply and/or metabolism of n-3 FA differs from that of the brain, and may more closely reflect liver secretion products. Although the retina is often regarded as a part of the brain, it is well known that the BRB and the BBB have differing permeability to a range of compounds (24,25). Differences in kinetics of essential FA retention and turnover may also explain these observations. The low DHA retina concentration in the 0.6 group is surprising and is evidence that n-6 FA are involved in retinal n-3 FA transport or metabolism.

Although LA and ALA were the dominant n-6 and n-3 FA in milk, AA and DHA were the principal FA in liver, plasma, brain, and retina at 30 d showing the pups have active enzyme systems for essential FA metabolism. For the pups, LA and AA were the primary n-6 liver and plasma FA, while the major n-6 brain and retina FA were AA and DPA, reflecting brain and retinal desaturation/elongation. Mouse brain and liver Δ-6-desaturase activity is highest during the brain growth spurt (41,42).

The dietary ratio of LA to ALA is a determining factor in the accretion of AA and DHA in fetal tissues (43) and infant erythrocytes (44), but thus far there is no clear evidence to indicate an optimal ratio for infant formula. Uauy *et al.* (8,45) proposed that the dietary ratio of total n-6 to n-3 FA should be maintained within a range of 5:1 to 15:1 during brain development. Bourre *et al.* (12) suggested the ratio range from 1 to 6 for rats. Van-Aerde *et al.* (46) emphasized that the dietary LA/ALA ratio and also the absolute level of LA and ALA should be taken into consideration and recommended that a formula range is between 5 to 15 by increasing ALA and decreasing LA content. In our study, a dietary LA/ALA ratio of 9 maintained retinal DHA at its highest levels, but we have no data indicating whether ratios closer to 1, with adequate LA, would increase retinal levels further, or indeed if the highest retinal DHA levels result in optimal function. Current practice for commercial formulas places the ratio of n-6/n-3 around 10 and preterm formulas are now available supplemented with AA and DHA. Further study is required to determine if there is an optimum ratio for human infants.

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Ultrasound-Assisted Oxidative Cleavage of Acetylenic and Ethylenic Bonds in Unsaturated Fatty Esters with Potassium Permanganate

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ABSTRACT: The effect of ultrasound on the oxidation of unsaturated fatty esters was investigated. Unsaturated fatty esters containing acetylenic or olefinic bonds or both were oxidatively cleaved with $KMnO_4$ in aqueous acetic acid ($AcOH/H_2O$, 1:1, vol/vol for olefinic fatty esters and $AcOH/H_2O$, 9:1, vol/vol for acetylenic fatty esters) to yield the corresponding carboxylic acid moieties in quantitative yields, when the reactions were conducted under ultrasound (20 kHz, 53 W/cm^2, 8–15 min). The methyl ester derivatives of the carboxylic acid moieties formed were identified by gas–liquid chromatographic analysis. The procedure constitutes a facile method for the determination of the position of the unsaturated center (olefinic or acetylenic) in an unsaturated fatty ester substrate, especially where the unsaturated system consists of an acetylenic bond.
Lipids 31, 1299–1301 (1996).

Ultrasound has increasingly been used to accelerate diverse types of chemical reactions and has become an established technique in organic synthesis (1–5). During the last few years, a large number of papers have appeared on the use of this technique, and excellent reviews are also available (6–10). In determining the position of an unsaturated center in long-chain fatty acids, oxidative cleavage of the olefinic bond is a common method used. The standard procedure described by von Rudloff (11), which employs sodium periodate in the presence of catalytic amount of potassium permanganate, has been widely used for this purpose. However, this method is not suitable for the determination of the position of a triple bond in acetylenic fatty acids, as a complex mixture of oxidized products is normally obtained (12). Acetylenic bonds are more resistant to oxidation than ethylenic bonds and require more vigorous oxidizing conditions. Even with solutions of permanganate or dichromate, the rate of oxidation of acetylenes is generally low unless certain phase-transfer catalysts (13,14) or solid supports (15) are used. The oxidation of alcohols (16) and arylalkanes (17) by solid $KMnO_4$ under concomitant ultrasonic irradiation has been shown to result in the production of carboxylic acids in high yield. We reason that under ultrasonic irradiation of $KMnO_4$ in a suitable solvent, ethylenic and acetylenic bonds may both be readily cleaved.

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Abbreviations: HRMS, high-resolution mass spectroscopy; NMR, nuclear magnetic resonance.

EXPERIMENTAL PROCEDURES

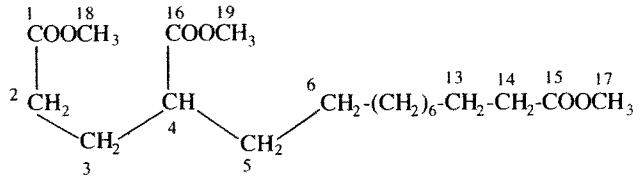
General procedure for the oxidative cleavage of methyl alkenoates as exemplified by the cleavage of methyl oleate (methyl 9Z-octadecenoate). A mixture of methyl oleate (0.21 g, 0.7 mmol), glacial acetic acid (10 cm^3), water (10 cm^3), and $KMnO_4$ (0.85 g, 5.38 mmol) was placed in a water-cooled jacketed glass cell. The reaction mixture was sonicated (Sonoreactor, 20 kHz, 53 W/cm^2, titanium horn; Undatim Ultrasonics, Louvain-la-Neuve, Belgium) for 8 min at 20°C. Aqueous sodium *meta*-bisulfite (10 cm^3, 5 M) was added. The reaction mixture was extracted with Et_2O (3 × 10 cm^3). The ethereal extract was washed with water (10 cm^3) and dried (Na_2SO_4). The filtrate was evaporated, and the residue was methylated by refluxing with BF_3-MeOH complex (14% w/w, 5 cm^3) in absolute MeOH (10 cm^3) for 10 min. Water (20 cm^3) was added to the reaction mixture, and the methyl ester derivatives were isolated by extraction with *n*-hexane (3 × 10 cm^3). The organic extract was washed with brine (5 cm^3) and dried (Na_2SO_4). The filtrate was evaporated under reduced pressure, and the weight of the residue was determined. Gas–liquid chromatographic analysis of the residue showed the presence of methyl nonanoate and methyl nonanedioate.

General procedure for the oxidative cleavage of methyl alkynoates as exemplified by the cleavage of methyl stearolate (methyl 9-octadecynoate). A mixture of methyl stearolate (0.21 g, 0.7 mmol), glacial acetic acid (18 cm^3), water (2 cm^3), and $KMnO_4$ (1.0 g, 6.8 mmol) was sonicated at 20°C for 15 min. Aqueous sodium *meta*-bisulfite (20 cm^3, 5 M) was added. The reaction mixture was extracted with Et_2O (3 × 10 cm^3). The ethereal extract was washed with water (10 cm^3) and dried (Na_2SO_4). The solvent was evaporated and the residue was methylated as described above. Gas Chromatographic analysis showed the presence of methyl nonanoate and methyl nonanedioate.

Oxidative cleavage of methyl hydnocarpate [methyl 11-(2-

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cyclopentenyl) undecanoate]. A mixture of methyl hydnocarpate (0.19 g, 0.7 mmol, isolated from *Hydnocarpus anthelminthica*) (18), glacial acetic acid (10 cm^3), water (10 cm^3), and $KMnO_4$ (0.95 g, 6 mmol) was sonicated at 20°C for 8 min. The reaction product was isolated and methylated as described above to give a triester. The structure of the triester was confirmed by nuclear magnetic resonance (NMR) spectroscopic analyses including two-dimensional ^{13}C-^1H and ^1H-^1H NMR COSY spectroscopy and high-resolution mass spectrometry (HRMS) (Scheme 1).



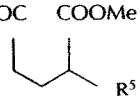
^1H NMR (δ_H) 1.3–1.6 (CH_2), 2.30 (*t*, J = 7.5 Hz, 14-*H*), 2.44 (*q*, J = 8.3 Hz, 3-*H*), 2.60 (quint., J = 7.9 Hz, 4-*H*), 2.72 (*t*, J = 6.4 Hz, 2-*H*), 3.66 (17-*H*, 18-*H*), 3.67 (19-*H*); ^{13}C-NMR (δ_C) 23.79 (C-5), 24.95 (C-13), 27.71 (C-3), 28.9-29.4, 34.09 (C-14), 37.02 (C-2), 44.74 (C-4), 51.43 (C-17, C-18), 51.75 (C-19), 173.33 (C-1, C-15), 174.30 (C-16); HRMS: $C_{19}H_{34}O_6$ calc. 358.9764; found 358.9777.

RESULTS AND DISCUSSION

Acidified potassium permanganate ($KMnO_4$) is normally used for the oxidative cleavage of alkenes to give the corresponding carboxylic acid moieties, while $KMnO_4$ under basic or neutral condition yields the corresponding diol derivatives (19). Considering the organic nature of the long-chain unsaturated fatty esters, aqueous acetic acid was considered the most suitable solvent system that would provide the required acidic environment in an organic phase for such reactions with $KMnO_4$ to succeed. Various mixtures of acetic acid and water were investigated to achieve the optimum condition. The results of the successful oxidative cleavage reactions of a number of ethylenic and acetylenic fatty esters with $KMnO_4$ in aqueous acetic acid under ultrasonic irradiation are presented in Table 1. A mixture of acetic acid/water (1:1, vol/vol) was used in the oxidative cleavage of the double bonds, while an acetic acid/water (9:1, vol/vol) was required for the successful cleavage of the triple bond in these unsaturated fatty esters. The same reactions conducted in pure (glacial) acetic acid were found to be incomplete even after 30 min of ultrasonic irradiation. The presence of water in the solvent system appeared therefore to be essential. Water is an extremely good conductor of ultrasonic waves and the effects of ultrasonic irradiation cause the reactants to be brought closely together through an efficient mixing or emulsification process. Reactions carried without ultrasonication were also found to be incomplete even after 12 h of stirring at ambient temperature. The need to increase the proportion of acetic acid in the solvent medium for the oxidation of acetylenic fatty esters was likely linked to the solubility of keto intermediates formed during the oxidation process. Also, the reaction time required to achieve complete oxidation of the triple bond in the acetylenic fatty esters was twice (15 min) as long as that needed for the double bond in olefinic fatty esters (8 min). These conditions reflect the higher bond strength of an acetylenic bond over that of an olefinic bond.

Oxidation of methyl oleate and methyl stearolate gave a

TABLE 1
Results of Oxidative Cleavage Reactions of Ethylenic and Acetylenic Fatty Esters with $KMnO_4$[a]

Substrate	Weight (g)	mmol	$KMnO_4$ g (mmol)	Product weight after methylation (g)	Relative percentages of components by gas chromatographic analyses (%)	
R^1-CH=CH-R^2	0.21	0.7	0.85 (5.40)[b]	0.27	R^1-COOMe	44
					MeOOC-R^2	56
R^3-CH=CH-CH_2-CH=CH-R^2	0.21	0.7	1.70 (10.8)[b]	0.18	R^3-COOMe	43
					MeOOC-R^2	57
H_2C=CH-R^4	0.14	0.7	1.26 (7.97)[b]	0.16	MeOOC-R^4	100
	0.19	0.7	0.94 (6.01)[b]	0.24		100
R^1-C≡C-R^2	0.21	0.7	1.07 (6.80)[c]	0.26	R^1-COOMe	47
					MeOOC-R^2	53
R^6-C≡C-C≡C-R^2	0.20	0.7	2.18 (13.8)[c]	0.22	R^6-COOMe	49
					MeOOC-R^2	51
R^6-CH=CH-C≡C-R^2 (*E*)	0.21	0.7	1.94 (12.3)[c]	0.23	R^6-COOMe	48
					MeOOC-R^2	52

[a]In aqueous acetic acid under ultrasonic irradiation. R^1 = $CH_3(CH_2)_7$; R^2 = $(CH_2)_7COOMe$; R^3 = $CH_3(CH_2)_4$; R^4 = $(CH_2)_8COOMe$; R^5 = $(CH_2)_{10}COOMe$; R^6 = $CH_3(CH_2)_5$.
[b]Solvent: AcOH/H_2O (1:1, vol/vol), 20 cm^3, 8 min ultrasonic irradiation.
[c]Solvent: AcOH/H_2O (9:1, vol/vol), 20 cm^3, 15 min ultrasonic irradiation.

mixture of nonanoic acid and nonanedioic acid. These carboxylic acid moieties, as their methyl esters, were readily identified by gas–liquid chromatographic analysis. Oxidation of methyl linoleate furnished nonanedioic acid and hexanoic acid. Succinic acid, one of the oxidation products, was not isolated from the reaction mixture due to its high solubility in water. The olefinic bond in methyl hydnocarpate was readily oxidized to yield a triester on esterification. Oxidation of a synthetic conjugated diacetylenic fatty ester (methyl 9,11-octadecadiynoate) gave heptanoic acid and nonanedioic acid. Oxidation cleavage of methyl santalbate [methyl (11*E*)-octadecen-9-ynoate, isolated from *Santalbum album* seed oil] (20) also furnished heptanoic acid and nonanedioic acid, which inferred the position of the conjugated enyne system between the C-9 and C-12 carbon atoms of the alkyl chain.

When these reactions were repeated under nonultrasound condition, none of these substrates could be completely oxidized in 12 h at room temperature as a complex mixture of products was obtained. The infrared spectrum of the crude products obtained from the nonultrasonic oxidation of methyl stearolate, for example, showed the presence of keto function (1710 cm^{-1}), which was confirmed by ^{13}C-NMR analysis (signal at 210 ppm). Gas chromatographic analysis of the ester derivatives of the product mixture indicated a range of components, which reflected the partial oxidation of the substrate

From the results of these experiments (Table 1), ultrasound-assisted oxidative cleavage of acetylenic and ethylenic fatty esters with $KMnO_4$ in aqueous acetic acid provides a means of determining the position of the unsaturated centers (double or triple bond) in the alkyl chain of the fatty ester.

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Preparation of Fatty Acid Methyl Esters from Lipoprotein and Macrophage Lipid Subclasses on Thin-Layer Plates

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ABSTRACT: A simple, accurate, and fast procedure for quantitative analysis of fatty acids (FA) in simple lipid subclasses from different biological specimens is presented. Lipid extracts of isolated plasma lipoproteins (very low, low, and high density lipoproteins; VLDL, LDL, and HDL, respectively) and permanent J774 mouse macrophages were fractionated into lipid subclasses by thin-layer chromatography (TLC) on silica gel 60 plates. Bands comigrating with authentic lipid standards were scraped off under argon and subjected to direct, *in situ* transesterification with BF_3/MeOH in the presence of the TLC adsorbent. Fatty acid methyl esters were subsequently quantitated by capillary gas chromatography. A comparison of the FA content present in total lipid extracts and in lipid subclasses separated by TLC revealed recoveries ranging from 93 (J774 cell extracts) to 99.7% (LDL). The method described is applicable for the measurement of FA in individual lipid subclasses and was successfully applied to quantitatively analyze the FA composition of the phospholipid, triacylglycerol, and cholesteryl ester fraction derived from VLDL, LDL, and HDL. In J774 lipid extracts, the FA composition of the phospholipid-, monoacylglycerol-, diacylglycerol-, free fatty acid-, triacylglycerol-, and cholesteryl ester fraction was quantitated. In addition we have analyzed the time-dependent loss of the major HDL polyunsaturated fatty acids (18:2, 20:4) in the phospholipid and cholesteryl ester fraction during copper-dependent peroxidation of HDL. We have not encountered analytical problems concerning low FA recoveries from CE-rich lipid extracts as indicated by almost quantitative recoveries of FA in LDL, HDL, and J774 extracts.
Lipids 31, 1303–1310 (1996).

The determination of free and esterified fatty acids (FA) in different biological tissues and fluids has been the target of numerous investigations (1). The quantitative measurement of the total FA content is a more or less straightforward procedure, provided that some precautions of utmost importance during sample homogenization and extraction are followed (for review see Ref. 2). Usually the FA components of lipids are converted to volatile derivatives prior to gas chromatography (GC) analysis, and methyl esters are the most popular among these although other derivatization procedures are used for special purposes (3). Many conventional methods used for GC analysis of FA derivatives are multistep procedures, involving multiple extraction and evaporation steps. Since these multistep procedures are sensitive to extraction losses, different one-step derivatization techniques improving recoveries of FA have been developed. Among the more commonly reagents used for the single-step formation of fatty acid methyl esters (FAME) are BF_3/MeOH (4,5) and acetyl chloride/methanol (6–9; and citation 14 in Ref. 4). In addition, methods circumventing extraction of lipids have been described for special purposes (10).

In contrast to the procedures described above, analysis of free fatty acids (FFA) present in different biological samples or analysis of the FA composition of different lipid subclasses is more cumbersome. It has been shown that direct procedures using acetyl chloride/methanol or diazomethane as methylating agents resulted in an overestimation of plasma FFA concentrations due to co-transesterification of small amounts of esterified FA (11,12). Therefore, a separate step for isolation of the FFA fraction before subsequent FA analysis was suggested. Lipid subclasses can be fractionated by a variety of different procedures but separation on prepacked solid-phase extraction (SPE) columns or sample fractionation by thin-layer chromatography (TLC) on silica gel 60 is the most commonly used procedure (13,14). When the latter method is chosen, lipids are either eluted from the TLC adsorbent prior to transesterification of the FA constituents (2) or, alternatively, can be transesterified directly on the TLC adsorbent (4). Direct transesterification of lipids on TLC sorbents was described for lipid subclasses obtained from pig liver (15), olive oil (16), spinach (17), human (18), and mouse plasma (12). One of the commonly observed disadvantages associated with this method was insufficient transesterification efficiency of cholesteryl esters (CE) in the presence of silica gel (15), although this problem could be circumvented by elution of CE with $CHCl_3$ prior to transesterification (15) or the use of Lewis acid as transmethylating agents (19,20).

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Abbreviations: acLDL, acetylated low density lipoprotein; BHT, butylated hydroxy toluene; CE, cholesteryl ester; DG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; FA, fatty acid; FAME, fatty acid methyl ester; FCS, fetal calf serum; FFA, free fatty acid; GC, gas chromatography; HDL, high density lipoproteins; LDL, low density lipoproteins; MG, monoacylglycerol; PBS, phosphate buffered saline; PL, phospholipid; PUFA, polyunsaturated fatty acid; SPE, solid-phase extraction; TG, triacylglycerol; TLC, thin-layer chromatography; VLDL, very low density lipoproteins.

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In addition, lipid/silica ratios higher than 4×10^3 were found to result in poor transesterification efficacy (17).

The aim of the present study was an investigation of the applicability of an *in situ* transmethylation procedure of free and esterified FA obtained from lipoprotein and cellular lipid subclasses adsorbed on silica gel 60. Our results demonstrate that the method described is useful for the determination of FA present in simple lipid subclasses obtained from lipoproteins and macrophages. Recoveries were almost quantitative, and we have observed no difficulties with CE-rich samples such as low density lipoprotein (LDL), high density lipoprotein (HDL), or lipid extracts obtained from lipid-laden macrophages. This is reflected by good agreement of the chemical composition of the various lipoprotein classes measured either enzymatically or calculated on the basis of the quantitative FA composition of the different lipid subclasses. The usefulness of the method is further demonstrated by good agreement of the sum of FA found in the individual lipid subclasses and the amount of FA present in the total lipid extracts.

MATERIALS AND METHODS

All organic solvents were of the highest purity available and purchased from Merck (Darmstadt, Germany). Extrelut columns were purchased from Merck. Monopalmitoyl-*rac*-glycerol, 1,2-dipalmitoyl-*sn*-glycerol, 1,3 dipalmitoyl-glycerol, tripalmitoyl-glycerol, phosphatidylcholine, cholesterol, cholesteryl-linoleate, heptadecanoic acid, butylated hydroxytoluene (BHT), and bovine serum albumin were obtained from Sigma (Deisenhofen, Germany). FAME reference mixtures (RM-3, PUFA-1, PUFA-2) were from Supelco (Vienna, Austria). J774 macrophages were obtained from ATCC (Rockville, MI). Dulbecco's modified Eagle's medium (DMEM) was purchased from Bio Whittaker (Antwerpen, Belgium), and fetal calf serum (FCS) was from Gibco (Vienna, Austria). PD-10 size exclusion columns were obtained from Pharmacia (Vienna, Austria). Enzymatic kits for phospholipid (PL), triacylglycerol (TG), CE, and cholesterol measurement were obtained from Boehringer Mannheim (Mannheim, Germany).

Lipid extraction. (i) Plasma lipoproteins. Lipoproteins of different hydrated density were isolated in a Beckman TLX-120 benchtop ultracentrifuge (Vienna, Austria) as described (21). Phosphate buffered saline (PBS, pH 7.4, 3.7 mL) were placed in the tube and underlayered with 1.7 mL of density adjusted plasma (1.24 g/mL, addition of 384 mg of solid KBr/mL plasma). The samples were spun for 2 h at 100.000 rpm (15°C). The very low density lipoprotein (VLDL), LDL, and HDL fractions were recovered by direct syringe aspiration of the lipoprotein bands and desalted by size-exclusion chromatography on PD-10 columns. PL, TG, CE, and cholesterol were quantitated with commercially available enzymatic kits according to the manufacturers' recommendations. Lipoprotein mass was quantitated on the basis of the chemical composition. Lipoprotein lipids (corresponding to 1 mg total lipoprotein) were extracted with $CHCl_3$/MeOH (2:1, Ref. 22), dried under nitrogen and redissolved in 500 µL $CHCl_3$. TLC and *in situ* transesterification were performed as described below.

(ii) Modified lipoproteins. HDL at a concentration of 1 mg total lipoprotein/mL was peroxidized in the presence of 64 µM Cu^{2+} (16 Cu^{2+} molecules/HDL particle) in O_2 saturated PBS. At the indicated time points, 1-mL samples were withdrawn to Pyrex tubes containing 1 mL of water (10 mg/mL EDTA) and 2 mL $CHCl_3$/MeOH, and lipids were extracted. Lipid extracts were concentrated on a TurboVapLV sample concentrator (Zymark, Idstein, Germany). The extracts were redissolved in 200 µL $CHCl_3$/MeOH and subjected to TLC prior to FA analysis of the lipid subclasses as described below. Acetylated LDL (acLDL) was prepared by multiple addition of 20 µL aliquots of acetic anhydride to LDL as described by Basu *et al.* (23).

(iii) Permanent J774 mouse macrophages. Adherent and confluent J774 macrophages (cultured on 75 cm^2 flasks in DMEM containing 10% FCS) were extracted twice with hexane/2-propanol (3:2, vol/vol; 5 mL, 20 min) on a plateau rotary shaker (24). Lipid extracts were dried under nitrogen and redissolved in 500 µL of $CHCl_3$. The protein content of the cellular residue was estimated according to the method of Lowry *et al.* (25). Prior to lipid analysis the cells were washed as described (24).

TLC separation of lipid extracts. To quantitate the FA composition of simple lipid subclasses, 200 or 300 µL aliquots of the lipid extracts originating from VLDL, LDL, HDL, and J774 macrophages were spotted on silica gel 60 plates under a gentle stream of argon. The plates were developed in a solvent system of hexane/diethyl ether (70:30, vol/vol) containing 1% acetic acid. The lipid bands were visualized with iodine vapor. Bands comigrating with authentic standards (≈ 10 µg/lane) were scraped off the TLC plates under an argon atmosphere in a plastic box and further processed as described below.

Preparation of FAMEs. (i) Total lipid extracts. Lipids were extracted from the various biological specimens in the presence of 100 µg heptadecanoic acid as internal standard and 500 µg BHT to prevent lipid oxidation. The organic layers were dried under a gentle stream of nitrogen and transesterified in the presence of 0.5 mL toluene and 1 mL of BF_3/MeOH (20%) at 110°C (90 min). Lipoprotein samples were freeze-dried in the presence of 100 µg internal standard (applied in $CHCl_3$ and dried under N_2) prior to transesterification of the dry residue (10). Excess BF_3 was destroyed by the addition of 2 mL of water and the FAME were extracted into 200 µL of hexane (final volume of the organic phase containing hexane and toluene was about 700 µL). Two µL were analyzed by GC as described below.

(ii) Transmethylation of individual lipid subclasses adsorbed on silica gel. The various lipid subclasses were scraped off the TLC plates and transferred to individual conical screw-capped glass tubes containing 50 µg of heptadecanoic acid and 250 µg BHT. *In situ* transesterification was performed with 0.5 mL toluene and 1 mL BF_3/MeOH in the presence of the TLC adsorbent at 110°C (90 min) without

prior extraction of the lipids. FAME were extracted into 200 μL of hexane, and 2 μL of the organic phase were analyzed by GC as described below.

Analysis of FAME by capillary GC. Separation of the FAME was performed on an HP-20M column (a nonbonded polyethyleneglycol phase; 25 m, 0.35 mm i.d.) using an HP 5890 gas chromatograph equipped with a flame-ionization detector and a split/splitless injector (Hewlett-Packard Co., Vienna, Austria). Helium was used as carrier gas, the split ratio was about 10:1. After an initial isothermal period of 5 min at 150°C, the temperature was programmed to 190°C at 4°/min and then to 215°C at 2°/min, with a hold at 215°C for 15 min. The detector temperature was 300°C; the injector was held at 300°C. Concentrations of the individual FA were calculated by peak area comparison with the internal standard. Data acquisition and calculation were performed with the Waters Millenium Software (Waters, Vienna, Austria). FA recovery was calculated from the amount of FA present in all of the simple lipid classes analyzed, and the FA concentration measured in the total lipid extract [% recovery = ($\Sigma FA_{PL,MG,DG,FFA,TG,CE}$/total FA · 100)]. In addition to these calculations, we have enzymatically measured the chemical composition of the different lipoprotein classes and compared these values with the chemical composition calculated on the basis of the FA content of the PL, TG, and CE fraction.

RESULTS AND DISCUSSION

TLC separation of lipids. Under the conditions described (hexane/diethyl ether, 70:30 containing 1% acetic acid) PL, MG, 1,2-DG, 1,3-DG, FFA, TG, and CE were clearly separated from each other. Only cholesterol and 1,2-DG comigrated in the mobile phase described above. The corresponding R_f values were: 0 (PL), 0.02 (MG), 0.15 (Cholesterol; 1,2-DG), 0.20 (1,3-DG), 0.29 (FFA), 0.61 (TG), and 0.71 (CE).

FA composition of lipoprotein lipid subclasses. The FA composition of total lipid extracts and the main lipid subclasses of autologous (i.e., lipoproteins isolated from the same plasma sample) VLDL, LDL, and HDL is shown in Tables 1–3. The majority of FA in VLDL is confined to the TG fraction, making up nearly 72% (1500 nmol/mg VLDL) of the total FA present in this particular lipoprotein (Table 1). CE and PL contained 9.6 and 18.8% (200.9 and 393.7 nmol/mg VLDL, respectively) of the remaining FA. As can be seen from Table 1, individual FA recovery ranged from 90 (22:6) to 105 (20:4) percentage. The predominant FA found in the PL fraction was 16:0. In the TG fraction 16:0 and 18:1 were the major individual FA while 18:2 was most abundant in the CE fraction. The PUFA/total FA ratio was 0.25:1 in the VLDL sample analyzed.

The FA compositions of LDL and the corresponding lipid subclasses are shown in Table 2. In accordance with the chemical composition of this lipoprotein about 55% of FA were found in the CE fraction (552.3 nmol/mg LDL corresponding to 1380 mol FA/mol LDL), while PL and TG contributed 33 and 13% of the remaining FA (343.4 and 129.1 nmol/mg LDL, respectively, corresponding to 860 and 320 mol FA/mol LDL). The recoveries for most individual FA ranged from 99 to 101%. Although all precautions were taken to avoid inadvertent oxidation of PUFA during sample workup, the recovery for 22:6 (accounting for only 0.7% of total LDL–FA) was lower (87%) compared to the other FA.

The FA content of total lipid extracts and lipid subclasses of total HDL (1.063–1.21 g/mL) was quantitated by GC. The results of these analyses are shown in Table 3. As can be seen from these data, the majority of FA was found in the PL (661 nmol/mg HDL) and CE (274.3 nmol/mg HDL) fraction. Recovery of the major FA was between 96–102%, while recoveries of 16:1, 18:1n-7, and 18:3 (contributing 0.2, 0.6, and 0.1% of total FA, respectively) were 64, 80, and 75%. PUFA (18:2, 20:4, and 22:6) constituted 34 and 63% of FA found in the PL and CE fraction, with 18:2 being the most abundant among PUFA. Overall, the total FA concentrations for VLDL, LDL, and HDL samples obtained during the present study were almost identical with our previous data (10). Furthermore, the percentage FA composition of lipoprotein lipid sub-

TABLE 1
Fatty Acid Composition of Very Low Density Lipoprotein (VLDL)[a]

Fatty acid	Fatty acid content in lipid subclasses (nmol/mg VLDL)			Total	Total fatty acids (nmol/mg VLDL)	Recovery (%)
	Phospholipids	Triglycerides	Cholesteryl esters			
16:0	133.2	476.6	31.1	640.9	674.6	95
16:1	5.3	71.3	10.2	86.8	91.4	95
18:0	63.6	69.3	6.5	139.4	142.2	98
18:1n-9	52.0	551.9	42.1	646.0	687.2	94
18:1n-7	7.1	46.3	3.2	56.6	56.7	100
18:2	83.5	250.5	95.3	429.3	447.2	96
18:3	1.9	16.8	1.5	20.2	20.4	99
20:4	39.2	12.7	9.9	61.8	58.9	105
22:6	7.9	4.4	1.1	13.4	14.9	90
Total	393.7	1499.8	200.9	2094.4	2193.5	95
Mol%	18.8	71.6	9.6	100.0		

[a]Results shown are mean values from duplicate analyses and are given in nmol/mg total lipoprotein.

TABLE 2
Fatty Acid Composition of Low Density Lipoprotein (LDL)[a]

Fatty acid	Fatty acid content in lipid subclasses (nmol/mg LDL)			Total	Total fatty acids (nmol/mg LDL)	Recovery (%)
	Phospholipids	Triglycerides	Cholesteryl esters			
16:0	120.4	38.2	73.2	231.8	229.1	101
16:1	2.5	4.5	21.7	28.7	29.1	99
18:0	54.8	10.2	6.1	71.1	69.4	102
18:1n-9	37.3	45.1	99.1	181.5	184.9	98
18:1n-7	5.5	2.4	6.4	14.3	15.0	95
18:2	75.8	23.2	303.9	402.9	405.5	99
18:3	0.9	1.4	3.5	5.8	5.8	100
20:4	38.9	3.2	36.1	78.2	76.8	102
22:6	7.3	0.9	2.3	10.5	12.1	87
Total	343.4	129.1	552.3	1024.8	1027.7	100
Mol%	33.5	12.6	53.9	100.0		

[a]Results shown are mean values from duplicate analyses and are given in nmol/mg total lipoprotein.

classes was similar to results published previously (14) where lipid subclasses were separated on NH_2(aminopropyl)-SPE columns.

Table 4 shows the chemical composition of the different lipoprotein samples obtained by enzymatic analysis with commercially available test kits. These data are compared with the chemical composition calculated from the amount of FA found in the corresponding lipid subclasses. The mean molecular masses used for these calculations were 800 (PL), 890 (TG), and 648 (CE), respectively. There was good agreement between measured and calculated values further supporting quantitative recovery of FA after direct transmethylation of simple lipid subclasses adsorbed on silica. For example, the HDL sample contained 25.5, 2.5, and 16.9% (w/w) PL, TG, and CE, in agreement with data reviewed (26). The corresponding calculated values were 26.4, 2.3, and 17.8%, respectively. It is noteworthy that also in the CE-rich lipoprotein samples (with exception of the LDL-PL fraction; 20 vs. 15%) there was good general agreement between enzymatic and calculated values. The difference in the calculated and enzymatically measured PL content of the LDL sample is presently not clear. It might be a reflection of the sphingomyelin content which could be accessible to enzymatic analysis but only partially accessible to BF_3/MeOH transesterification.

We have also analyzed the FA content of the various lipid subclasses in the corresponding human plasma sample (data not shown). In plasma the recoveries of individual FA were in the range of 91 (18:2) to 119 (16:1) percentage. The FFA concentration in the plasma sample was 1.06 mM, in good agreement with other reports (18,27) but higher compared to data reported by Hallaq *et al.* (0.092 mM, Ref. 11).

PUFA consumption during Cu^{2+} mediated lipid peroxidation of HDL. Recent findings have emphasized the role of HDL as the major carrier of circulating lipid hydroperoxides, acting as a sink for these potentially proatherogenic lipids (28). We have been interested in whether *in situ* transmethylation of lipid subclasses might be both a suitable and a simple analytical tool to follow PUFA consumption in the various lipid subclasses during Cu^{2+} initiated lipid peroxidation. At the time points indicated, samples were withdrawn, extracted with $CHCl_3$/MeOH, and PL, TG, and CE were separated by TLC as described in the Materials and Methods section. Silica adsorbed lipids were directly transmethylated, and

TABLE 3
Fatty Acid Composition of High Density Lipoprotein (HDL)[a]

Fatty acid	Fatty acid content in lipid subclasses (nmol/mg HDL)			Total	Total fatty acids (nmol/mg HDL)	Recovery (%)
	Phospholipids	Triglycerides	Cholesteryl esters			
16:0	243.6	38.6	37.6	319.8	332.5	96
16:1	2.5	n.d.	n.d.	2.5	3.9	64
18:0	95.4	n.d.	2.7	98.1	99.2	99
18:1n-9	93.0	15.5	54.4	162.9	159.3	102
18:1n-7	n.d.	n.d.	6.4	6.4	8.0	80
18:2	195.6	25.3	147.0	367.9	370.9	99
18:3	0.9	n.d.	n.d.	0.9	1.2	75
20:4	30.0	1.1	26.2	57.3	56.8	101
22:6	n.d.	n.d.	n.d.	n.d.	n.d.	
Total	661.0	80.5	274.3	1015.8	1031.8	98
Mol%	65.1	7.9	27.0	100.0		

[a]Results shown are mean values from duplicate analyses and are given in nmol/mg total lipoprotein; n.d., not detectable.

TABLE 4
Comparison of the Chemical Composition of the Different Lipoproteins[a]

		Chemical composition (%, w/w)				
		Phospholipids	Triglycerides	Cholesteryl esters	Cholesterol	Protein
VLDL	enz.[b]	17.5	47.6	13.9	6.4	14.7
	FA[c]	16.1	44.5	13.0		
LDL	enz.[b]	20.0	4.5	38.7	9.5	27.0
	FA[c]	14.9	4.0	36.0		
HDL	enz.[b]	25.5	2.5	16.9	3.0	52.0
	FA[c]	26.4	2.3	17.8		

[a]Measured enzymatically and calculated on the basis of the fatty acid (FA) composition of the various lipid subclasses; VLDL, very low density lipoprotein; see Tables 1 and 2 for other abbreviations.
[b]Measured enzymatically as indicated in the Materials and Methods section.
[c]Calculation based on the FA composition of the different simple lipid subclasses of the corresponding lipoprotein.

the time-dependent loss of 18:2 and 20:4 was analyzed. PUFA consumption in the CE and PL fraction as a consequence of Cu^{2+} mediated lipid peroxidation is shown in Figure 1. Oxidation of 20:4 started almost immediately after addition of Cu^{2+} without an apparent lag phase. Oxidation of CE and PL associated 18:2 proceeded at somewhat slower rates compared to 20:4 peroxidation. There were, however, almost no differences in the (initial) oxidation rates estimated for the PL and CE fraction (3 and 2.3 nmol $mg^{-1}min^{-1}$). The observation that there is no apparent delay between surface (PL) and core (CE) PUFA oxidation is in line with results obtained during one of our earlier studies (29) where HDL peroxidation was initiated with a peroxyl-radical generating azo-compound (AAPH). In that study (29), lipid extracts were analyzed by HPLC with chemiluminescence detection, and it is noteworthy, that the (relatively) simple equipment (i.e., *in situ* transmethylation of lipids adsorbed on thin-layer adsorbents and subsequent GC analysis of FAME) used in the present study yielded comparable results.

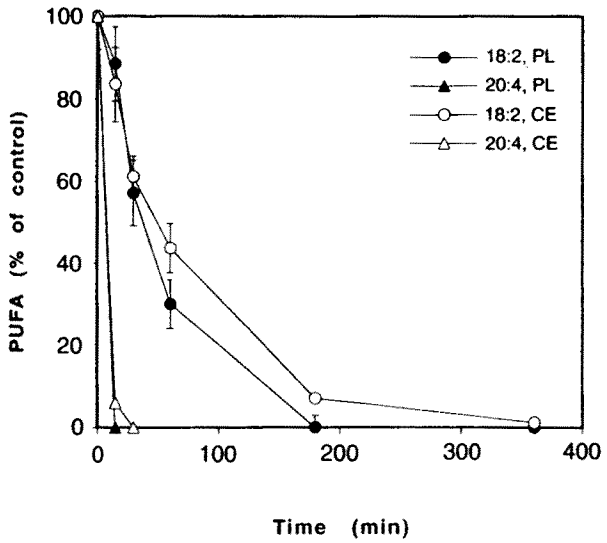


FIG. 1. Loss of high density lipoprotein (HDL)-polyunsaturated fatty acids (PUFA) during Cu^{2+}-mediated peroxidation. HDL at a concentration of 1 mg total lipoprotein/mL was peroxidized in the presence of 64 μM Cu^{2+} (16 Cu^{2+} molecules/HDL particle) in O_2 saturated phosphate buffered saline. At the indicated time points, 1 mL samples of the incubation mixture were withdrawn, extracted as described in the Materials and Methods section and subjected to thin-layer chromatography separation. Phospholipid (PL)- and cholesteryl ester (CE)-fatty acids were transesterified with BF_3/MeOH and analyzed by capillary gas chromatography. Values are given as the mean ± SD from triplicate analysis.

FA composition of J774 macrophages. Oxidation or modification of LDL by reactive lipid peroxidation products is thought to be an initiating event during atherogenesis (30), and evidence from *in vivo* studies suggest that modified LDL is present in atherosclerotic plaques (31,32). J774 macrophages are used in many laboratories to investigate mechanisms leading to the formation of lipid-laden foam cells (32,33). These studies are routinely performed using radioactively labeled lipoproteins. Here we intended to investigate if the method presented allows accurate measurements of differences in the cellular FA composition as a result of modified lipoprotein uptake.

In Table 5 the total cellular FA composition and the FA composition of individual lipid subclasses of J774 macrophages are presented. The majority of FA was found in the PL fraction (222 nmol/mg cell protein, 71% of total FA) with 16:0, 18:1, and 20:4 as the predominant FA (27, 25, and 14% of the total PL–FA, respectively). The FFA fraction in J774 macrophages was almost exclusively constituted by saturated FA, i.e., 14:0, 16:0, and 18:0, and this might reflect the steady-state concentrations of cellular FA synthesis. Calculated on a percentage basis, the FA compositions of PL, MG, DG, TG, and CE were similar. Recoveries for cellular 20:4, 20:5, and 22:6 were somewhat lower (85–92%) as compared to the other FA identified in the same samples (89–107%).

It is a well-known feature of J774 macrophages that they acquire large amounts of intracellular lipid when cultured in the presence of acLDL (33). In line with these observations, incubation of J774 cells in the presence of acLDL (20 mg/75 cm^2) led to remarkable changes in the amount and composition of cellular FA. Cultivation of J774 macrophages in the presence of acLDL increased the amount of total cellular FA from 322 to 633 nMol/mg cell protein (Table 5 vs. Table 6,

TABLE 5
Fatty Acid Composition of J774 Macrophages Cultured in the Absence of Acetylated Low Density Lipoprotein[a]

Fatty acid	Fatty acid content in lipid subclasses (nmol/mg cell protein)							Total fatty acids (nmol/mg cell protein)	Recovery (%)
	Phospholipids	Monoglycerides	Diglycerides	Free fatty acids	Triglycerides	Cholesteryl esters	Total		
14:0	11.3 ± 1.21	0.3 ± 0.00	0.9 ± 0.00	2.9 ± 0.00	1.4 ± 0.05	1.0 ± 0.01	17.9 ± 1.27	17.9 ± 0.04	100
16:0	60.3 ± 3.00	1.8 ± 0.06	5.4 ± 0.53	20.5 ± 1.97	5.0 ± 0.25	2.5 ± 0.05	95.5 ± 5.85	89.3 ± 5.41	107
16:1	23.7 ± 1.20	0.0 ± 0.01	0.6 ± 0.00	0.6 ± 0.01	1.0 ± 0.06	0.6 ± 0.01	26.5 ± 1.28	26.7 ± 1.00	99
18:0	23.6 ± 1.50	1.3 ± 0.12	1.7 ± 0.23	24.9 ± 2.23	2.0 ± 0.16	1.4 ± 0.02	54.9 ± 4.27	54.7 ± 4.23	100
18:1	55.6 ± 4.00	0.6 ± 0.07	1.8 ± 0.18	2.2 ± 0.06	3.1 ± 0.42	1.5 ± 0.05	64.8 ± 4.77	72.0 ± 3.76	90
18:2	8.6 ± 0.65	0.2 ± 0.10	0.6 ± 0.02	n.d.	0.4 ± 0.30	0.4 ± 0.07	10.2 ± 1.14	11.5 ± 0.94	89
18:3	3.0 ± 0.04	0.1 ± 0.00	0.4 ± 0.00	0.4 ± 0.01	0.2 ± 0.01	0.1 ± 0.01	4.2 ± 0.06	5.4 ± 1.39	77
20:4	30.1 ± 1.00	n.d.[b]	0.3 ± 0.01	0.3 ± 0.05	0.5 ± 0.10	0.1 ± 0.01	31.4 ± 1.17	36.7 ± 3.31	85
20:5	1.8 ± 0.06	n.d.	n.d.	n.d.	n.d.	n.d.	1.8 ± 0.06	2.1 ± 0.07	88
22:6	4.5 ± 0.15	n.d.	n.d.	n.d.	0.8 ± 0.01	n.d.	5.3 ± 0.16	5.8 ± 0.28	92
Total	222.6 ± 12.80	4.3 ± 0.37	11.7 ± 0.97	51.7 ± 4.33	14.4 ± 1.35	7.6 ± 0.21	312.4 ± 20.04	322.1 ± 20.44	93
Mol%	71.3	1.4	3.7	16.6	4.6	2.4	100.0		

[a]Results represent mean ± SD of three different 75 cm^2 flasks (containing 3–5 mg cell protein).
[b]Not detectable (<100 pMol).

Fig. 2) and this was also reflected by the FA composition of simple lipid subclasses. While PL– and TG–FA increased moderately by 1.4- and 3.7-fold, respectively, the FA content in the CE fraction increased almost 22-fold (7.6 and 210 nMol/mg cell protein, untreated vs. treated cells, respectively, Table 5, Table 6 and Fig. 2). The percentage FA composition of the cellular CE fraction was altered upon incubation of J774 macrophages with acLDL: While 18:0 and 18:1 contributed 15 and 16% in control cells (incubated without acLDL), these FA accounted for 32 and 26%, respectively, in cells incubated in the presence of acLDL. Concommitantly the FA concentration present in the minor lipid subclasses (MG and DG) increased 6- and 13-fold, respectively. In parallel to the increase in FA located in PL, MG, DG, TG, and CE, FFA were decreased by 66% (from 52 to 16.2 nMol/mg cell protein).

A calculation of the absolute increase in FA concentrations observed in the PL, TG, and CE fraction revealed that the increase in cellular FA closely reflected the chemical composition of an LDL particle: The overall increase of 311 nMol/mg cell protein in total cellular FA (Table 5 vs. Table 6) would correspond to an internalization of about 280 µg acLDL. According to the chemical composition of this lipoprotein, an increase by 21, 135, and 180 nMol FA/mg cell protein in the cellular PL, TG, and CE fraction would be expected. In fact we have observed that the FA content in PL, TG, and CE was increased by 39, 95, and 200 nMol/mg cell protein, respectively. These findings suggest that acLDL associated lipids are not very actively metabolized by J774 cells; however, a detailed time course is necessary to clarify this question.

Summarizing our results, we have shown that preseparation of lipid extracts obtained from different biological specimens into the corresponding lipid subclasses by TLC followed by *in situ* transmethylation of lipids adsorbed to silica is applicable to a variety of different analytical topics in the field of lipid/lipoprotein analysis. Under the conditions described, we have not encountered problems with CE-rich samples, best reflected by the high recoveries during HDL

TABLE 6
Fatty Acid Composition of J774 Macrophages Cultured in the Presence of 20 mg Acetylated Low Density Lipoprotein[a]

Fatty acid	Fatty acid content in lipid subclasses (nmol/mg cell protein)							Total fatty acids (nmol/mg cell protein)	Recovery (%)
	Phospholipids	Monoglycerides	Diglycerides	Free fatty acids	Triglycerides	Cholesteryl esters	Total		
14:0	3.9 ± 0.98	n.d.	n.d.	0.5 ± 0.01	1.4 ± 0.12	8.4 ± 0.87	14.2 ± 1.98	15.7 ± 1.20	100
16:0	91.6 ± 4.10	1.1 ± 0.06	3.8 ± 0.83	5.9 ± 0.67	21.7 ± 0.25	51.5 ± 0.98	175.6 ± 6.89	173.3 ± 9.39	107
16:1	13.0 ± 0.91	n.d.	0.6 ± 0.20	0.2 ± 0.01	2.6 ± 0.10	14.0 ± 1.11	30.4 ± 2.33	29.1 ± 1.30	99
18:0	48.8 ± 3.20	0.7 ± 0.09	1.3 ± 0.23	7.0 ± 1.11	5.6 ± 0.19	6.3 ± 0.04	69.7 ± 4.86	60.1 ± 4.95	100
18:1	64.4 ± 2.51	1.0 ± 0.07	2.4 ± 0.22	2.1 ± 0.02	16.2 ± 0.82	67.3 ± 4.70	153.4 ± 8.34	169.6 ± 15.20	90
18:2	55.2 ± 0.58	0.2 ± 0.03	0.9 ± 0.15	0.5 ± 0.04	4.8 ± 0.43	54.7 ± 0.62	116.3 ± 1.85	134.6 ± 8.31	89
18:3	n.d.[b]	n.d.	n.d.	n.d.	0.3 ± 0.01	1.4 ± 0.01	1.6 ± 0.01	1.6 ± 0.00	77
20:4	22.1 ± 1.91	n.d.	n.d.	n.d.	0.3 ± 0.11	3.1 ± 0.01	25.5 ± 2.03	25.6 ± 1.19	85
20:5	3.8 ± 0.06	n.d.	n.d.	n.d.	n.d.	0.5 ± 0.00	4.3 ± 0.06	4.9 ± 0.50	88
22:6	14.4 ± 1.21	n.d.	1.1 ± 0.10	n.d.	n.d.	3.0 ± 0.00	18.5 ± 1.31	18.7 ± 0.33	92
Total	317.2 ± 15.46	3.0 ± 0.25	10.1 ± 1.73	16.2 ± 1.86	52.9 ± 2.02	210.2 ± 8.33	609.5 ± 29.65	633.2 ± 42.37	93

[a]Results represent mean ± SD of three different 75 cm^2 flasks (containing 3.5–6 mg cell protein).
[b]Not detectable (<100 pMol)

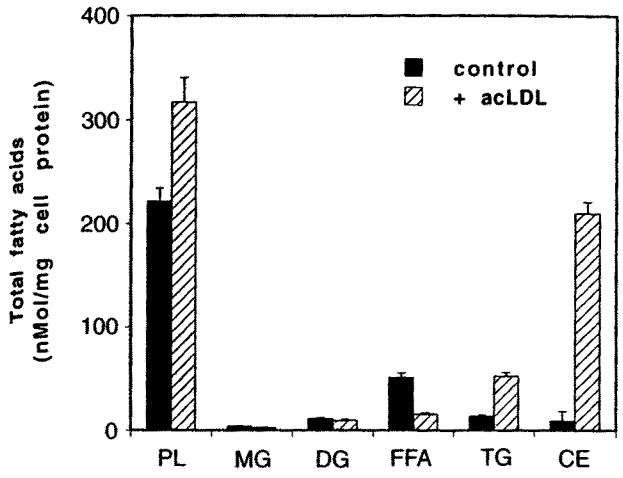


FIG. 2. Total fatty acid composition of lipid subclasses isolated from J774 cells grown in the absence (controls) or in the presence [+ acetylated low density lipoprotein (acLDL)] of 20 mg acLDL. Confluent cells were used for the experiments. Cell culture, lipid extraction, thin-layer chromatography separation, and gas chromatographic analysis of cellular lipids were performed as described in the Materials and Methods section. Values are given as the mean ± SD from triplicate experiments; PL, phospholipid; MG, monoacylglycerol; DG, diacylglycerol; FFA, free fatty acid; TG, triacylglycerol; CE, cholesteryl ester.

and LDL analysis, lipoproteins in which the lipid domain is comprised of 30 and 50% CE, respectively. Despite the simplicity of the method, accurate and reproducible data with high recoveries for individual FA are obtained.

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Analysis of Triacylglycerols by Silver-Ion High-Performance Liquid Chromatography–Atmospheric Pressure Chemical Ionization Mass Spectrometry

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ABSTRACT: Triacylglycerols of the seed oils rich in α- and/or γ-linolenic acid moieties were separated by silver-ion high-performance liquid chromatography (HPLC) followed by on-line atmospheric pressure chemical ionization–mass spectrometric (APCI–MS) detection. Mass spectra of most triacylglycerols exhibited abundant $[M + H]^+$ and $[M - RCO_2]^+$ ions, which defined the molecular weight and the molecular association of fatty acyl residues of a triacylglycerol, respectively. Silver ions formed weaker complexes with triacylglycerols containing γ-linolenic acid than with those containing α-linolenic acid, i.e., the elution order of molecules was $XYT_\gamma > XYT_\alpha$, $XT_\gamma T_\gamma > XT_\gamma T_\alpha > XT_\alpha T_\alpha$, and $T_\gamma T_\gamma T_\gamma > T_\gamma T_\gamma T_\alpha > T_\gamma T_\alpha T_\alpha > T_\alpha T_\alpha T_\alpha$, where T_α = α-linolenic acid, T_γ = γ-linolenic acid, and X, Y = fatty acids different from linolenic acid. Furthermore, silver-ion HPLC resulted in partial separation within equally unsaturated triacylglycerols according to differences in the combined number of acyl carbons. Regioisomeric forms of triacylglycerols were not determined from the seed oil samples, although differences were measured with reference compounds in the relative abundances of $[M - RCO_2]^+$ ions formed by a loss of a fatty acyl residue from the *sn*-2 position and the *sn*-1/3 positions. Silver-ion HPLC/APCI–MS provided valuable information for structure elucidation of seed oil triacylglycerols: 43 molecular species were identified from cloudberry seed oil, 39 from evening primrose oil, 79 from borage oil, 44 from alpine currant, and 56 from black currant seed oils. The quantitation requires to be studied further, especially in those cases where several molecular weight species of triacylglycerols eluted in a single chromatographic peak.

Lipids 31, 1311–1322 (1996).

Silver-ion chromatography is a practical technique for the separation of triacylglycerols according to differences in unsaturation (reviewed in Refs. 1–4). Previous studies have been performed by thin-layer chromatography (TLC) on silica gel plates impregnated with silver ions. More recent studies are mostly based on high-performance liquid chromatographic (HPLC) separation either on a reversed-phase column (silver ions in the mobile phase) (5) or on a silver-loaded cation-exchange column (6), or on a silver-loaded silica column (7). In general, the separation of triacylglycerols on silver-ion chromatography is based on (i) the number of double bonds in the acyl chains, (ii) the distribution of double bonds between the fatty acyl residues within a single molecule, (iii) the configuration and position of double bonds within a fatty acyl residue, and (iv) the position at which the fatty acid is esterified to the glycerol backbone.

The method introduced by Christie (8), based on separation on a silver-loaded cation-exchange column, has been most widely used and applied to the analysis of various samples, such as seed and vegetable oils (8–11), milk fat (12–14), and fish oils (15–17). The technique has not yet been used for the analysis of samples containing both α-linolenic acid (18:3n-3) and γ-linolenic acid (18:3n-6) moieties in their triacylglycerols, although resolution of ester derivatives of α- and γ-linolenic acids has been reported (18). Few studies of oils rich in γ-linolenic acid have been conducted by silver-ion chromatography, for example, evening primrose oil by silver-ion HPLC (19) and silver-ion TLC (20), and borage oil by silver-ion supercritical fluid chromatography (21). Triacylglycerols of the seeds of the plants belonging to the genus *Ribes* are relatively abundant both in γ- and α-linolenic acids. The composition of black currant seed oil has been studied, for example, by reversed-phase HPLC (22–24), high-temperature gas chromatography (GC) (24), and direct inlet chemical ionization-mass spectrometry (MS) (24,25). Recently, a separation of α- and γ-linolenic acid containing triacylglycerols with an identical number of acyl carbons and degree of unsaturation has been obtained by capillary supercritical fluid chromatography (25). However, the differentiation of co-eluting components was not possible with a flame-ionization detector (FID).

Components separated by silver-ion HPLC are most often detected with an evaporative light-scattering detector (ELSD) or with an FID, which, unfortunately, do not produce structural information on molecular compositions. Therefore, identification of the components has been done either by collection of the separated HPLC fractions and analysis of their

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Abbreviations: APCI, atmospheric pressure chemical ionization; APCI–MS, atmospheric pressure chemical ionization–mass spectrometry; ELSD, evaporative light-scattering detector; FID, flame-ionization detector; HPLC, high-performance liquid chromatography; GC, gas chromatography; MS, mass spectrometry; TLC, thin-layer chromatography; RIC, reconstructed ion chromatogram.

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fatty acid compositions as methyl esters by GC, or by comparison of the retention times of unknown peaks with those of reference components. An application of an on-line combination of argentation HPLC, on a silver-loaded silica column, with MS utilizing electrospray ionization has been reported (26). Mass spectra of triacylglycerols exhibited only abundant $[M + Na]^+$ ions without information on fatty acid moieties. Information on both the molecular weights and the fatty acyl residues of triacylglycerols has been achieved by combining reversed-phase HPLC separation with atmospheric pressure chemical ionization–mass spectrometry (APCI–MS) (27,28). Mass spectra of most triacylglycerols consisted of abundant $[M + H]^+$ and $[M - RCO_2]^+$ ions.

The objectives of this study were to apply silver-ion HPLC for the separation of α- and γ-linolenic acid containing triacylglycerols in seed oils and APCI–MS for identification purposes. Chromatographic separation of triacylglycerols containing α- and γ-linolenic acid moieties is essential, because double-bond positional isomers cannot be distinguished as such by MS.

EXPERIMENTAL PROCEDURES

Materials. Oils from the seeds of black currant (*Ribes nigrum*), alpine currant (*R. alpinum*), and cloudberry (*Rubus chamaemorus*) were extracted with supercritical carbon dioxide at Flavex (Rehlingen, Germany). Evening primrose oil (*Oenothera biennis*) and borage oil (*Borago officinalis*) were commercial products purchased from a local health food shop. The oils were dissolved in *n*-hexane, after which the triacylglycerols were purified by elution through a short column of Florisil™ (Fluka Chemie AG, Buchs, Switzerland) with 10 mL *n*-hexane/diethyl ether (4:1, vol/vol). After evaporating the solvent with a stream of nitrogen, the triacylglycerols were dissolved in 1,2-dichloroethane to a concentration of ~10 mg/mL and stored under nitrogen at –20°C. All solvents used were HPLC-grade supplied by Rathburn (Walkerburn, Scotland) or Merck (Darmstadt, Germany). The triacylglycerol standards *rac*-1,2-distearoyl-3-palmitoyl-*sn*-glycerol (*rac*-18:0-18:0-16:0), 1,3-distearoyl-2-palmitoyl-*sn*-glycerol (*sn*-18:0-16:0-18:0), *rac*-1,2-dipalmitoyl-3-oleoyl-*sn*-glycerol (*rac*-16:0-16:0-18:1), 1,3-dipalmitoyl-2-oleoyl-*sn*-glycerol (*sn*-16:0-18:1-16:0), *rac*-1,2-dioleoyl-3-palmitoyl-*sn*-glycerol (*rac*-18:1-18:1-16:0), 1,3-dioleoyl-2-palmitoyl-*sn*-glycerol (*sn*-18:1-16:0-18:1), *rac*-1,2-dipalmitoyl-3-linoleoyl-*sn*-glycerol (*rac*-16:0-16:0-18:2), 1,3-distearoyl-2-linoleoyl-*sn*-glycerol (*sn*-18:0-18:2-18:0), *rac*-1,2-dioleoyl-3-γ-linolenoyl-*sn*-glycerol (*rac*-18:1-18:1-18:3n-6), 1,3-dioleoyl-2-γ-linolenoyl-*sn*-glycerol (*sn*-18:1-18:3n-6-18:1), 1,3-dioleoyl-2-α-linolenoyl-*sn*-glycerol (*sn*-18:1-18:3n-3-18:1), trilinoleoylglycerol (18:2-18:2-18:2), and trilinolenoylglycerol (18:3n-3-18:3n-3-18:3n-3) were purchased from Larodan (Malmö, Sweden).

GC. Fatty acid methyl esters were prepared by sodium methoxide-catalyzed transesterification of the purified triacylglycerol fractions of the seed oils (29). Separations of methyl esters on an NB-351 fused silica capillary column (25 m × 0.32 mm i.d. with 0.20 μm film; HNU-Nordion Ltd., Helsinki, Finland) were conducted with a Varian 3300 gas chromatograph (Limerick, Ireland) equipped with a split injector (split ratio 40:1) and an FID. The temperature program was from 120°C (2 min) with a rate of 3°C/min to 230°C (10 min). The injector temperature was held at 225°C and the detector at 240°C. Helium was used as a carrier gas.

Silver-ion HPLC. Two different HPLC systems were used: (i) a Merck Hitachi L-6200A Intelligent pump (Hitachi, Tokyo, Japan), equipped with an SSI Model LP-21 lo-pulse unit (Scientific Systems Inc., State College, PA), combined with mass spectrometric detection, and (ii) a Shimadzu LC-9A pump (Kyoto, Japan), equipped with a Shimadzu FCV-9AL low-pressure gradient elution unit, combined with an ELSD (Cunow DDL21; Cergy-Saint-Christophe, France).

Silver-ion HPLC was performed according to the procedure introduced by Christie (6,8). A cation-exchange column containing sulfonic acid moieties (ET 250/4 Nucleosil 100-5 SA, 250 mm × 4 mm i.d., 5 μm particle size; Macherey-Nagel, Düren, Germany) was loaded with silver ions. Triacylglycerols in 1,2-dichloroethane (~5 μL) were injected onto the column and separated at ambient temperature using a ternary solvent gradient consisting of A) dichloromethane/1,2/dichloroethane (1:1, vol/vol), B) acetone, and C) acetone/acetonitrile (4:1, vol/vol). The program steps of the linear gradient were: 0 min 100% A; 10 min 50% A–50% B; 30 min 70% B–30% C; 60 min 100% C; 70 min 100% C. The flow rate was 0.8 mL/min. The column was equilibrated for 20 min at the initial conditions before each analysis.

MS. The MS determinations were conducted with a Finnigan MAT TSQ-700 instrument (San Jose, CA) equipped with an APCI source and an ICIS II data system. The MS parameters were optimized for the ionization of triacylglycerols using loop injection technique. During the HPLC–MS analyses, the vaporizer temperature was 400°C, capillary heater temperature 200°C, corona current 5 μA, sheath gas (nitrogen) pressure 50 psi, and auxiliary gas (nitrogen) flow 5 mL/min. Positively-charged ions with *m/z* values of 400–1100 were scanned with a scan time of 0.7 s. The HPLC flow of 0.8 mL/min was introduced into the APCI source without any splitting.

RESULTS

Fatty acid compositions. The samples studied were selected to represent seed oils containing (i) only α-linolenic acid, (ii) only γ-linolenic acid, and (iii) both α- and γ-linolenic acids (Table 1). Cloudberry seed oil was the example of an oil rich in α-linolenic acid (28 mol%). Evening primrose oil and borage oil were the examples of γ-linolenic acid oils. Borage oil contained 22 mol% γ-linolenic acid and evening primrose oil 10 mol%. The proportion of α-linolenic acid in these oils was negligible. The seed oils of black currant and alpine currant contained significant proportions of both linolenic acid isomers. Black currant seed oil contained 14 mol% both

TABLE 1
Fatty Acid Compositions (mol%) of the Triacylglycerols of Cloudberry, Evening Primrose, Borage, Alpine Currant, and Black Currant Seed Oils

	Cloudberry		Evening primrose		Borage		Alpine currant		Black currant	
Fatty acid	Mean[a]	SD	Mean[a]	SD	Mean[a]	SD	Mean[a]	SD	Mean[a]	SD
14:0	0.41	0.02								
16:0	3.15	0.07	6.17	0.08	10.76	0.28	5.55	0.07	5.72	0.10
16:1n-7	0.41	0.02		0.16	0.00					
16:1n-5	0.46	0.01		0.16	0.01					
18:0	1.36	0.00	1.38	0.02	3.61	0.03	1.40	0.02	1.59	0.02
18:1n-9	14.94	0.05	9.09	0.03	16.51	0.02	19.25	0.06	13.49	0.05
18:1n-7	0.64	0.02	0.67	0.01	0.65	0.01	0.94	0.03	0.75	0.01
18:2n-6	40.23	0.16	72.02	0.07	37.19	0.05	41.10	0.08	45.96	0.03
18:3n-6			9.62	0.02	22.41	0.12	9.18	0.03	14.24	0.03
18:3n-3	28.24	0.07	0.21	0.00	0.13	0.00	17.79	0.01	13.81	0.02
18:4n-3					0.13	0.01	3.41	0.01	2.92	0.01
20:0	1.11	0.02	0.25	0.01	0.22	0.02			0.29	0.01
20:1n-9	0.74	0.03	0.17	0.01	3.92	0.13			0.87	0.01
20:2n-6	4.42	0.12			0.18	0.01				
22:0	0.51	0.00			0.14	0.02				
22:1n-9					2.37	0.13				
22:2	2.02	0.06								
24:1n-9					1.34	0.09				
Others	1.38	0.29	0.42	0.03	0.11	0.04	1.39	0.05	0.36	0.02

[a]Average of three gas chromatographic analyses of fatty acid methyl esters.

γ-linolenic and α-linolenic acid, whereas the corresponding proportions for alpine currant oil were 9 and 18 mol%, respectively. An additional feature of the seed oils of plants belonging to the genus *Ribes* was the presence of stearidonic acid (18:4n-3). All the oils studied were abundant in oleic acid (9–19 mol%) and linoleic acid (37–72 mol%).

APCI–MS of triacylglycerols. APCI of most triacylglycerols yielded very simple mass spectra exhibiting abundant $[M + H]^+$ and $[M - RCO_2]^+$ ions, which defined the molecular weight and the molecular association of fatty acyl residues, respectively. As reported earlier by Byrdwell and Emken (27) and Neff and Byrdwell (28), the relative abundances of $[M + H]^+$ and $[M - RCO_2]^+$ ions were strongly affected by the degree of unsaturation of a triacylglycerol, e.g., saturated molecules did not produce any $[M + H]^+$ ion, whereas $[M + H]^+$ ion was the base peak in the mass spectra of more unsaturated triacylglycerols (typically the combined number of double bonds in the acyl chains is ≥4). During the optimization of the MS parameters, it was observed that the ionization efficiency of triacylglycerols decreased with increasing capillary temperature. However, the capillary temperature had to be high enough to produce a relatively low and stable signal background. Therefore, the practical capillary temperature, with the instrumentation and eluent composition used, was in the range of 170–200°C. The vaporizer temperature had to be high enough to produce good sensitivity, the optimum being around 400°C.

Silver-ion HPLC/APCI–MS of reference components. It was shown by Nikolova-Damyanova *et al.* (18) that methyl and phenacyl esters of γ-linolenic acid were less retained on a silver-loaded cation-exchange column than those of α-linolenic acid. This let us to suggest that it may also be possible to separate triacylglycerols containing α- and γ-linolenic acid moieties with the technique. Figure 1 shows the separation of 1,3-dioleoyl-2-γ-linolenoyl-*sn*-glycerol and 1,3-dioleoyl-2-α-linolenoyl-*sn*-glycerol by HPLC on a silver-loaded Nucleosil-5 SA column. The interaction of γ-linolenic acid containing isomer with silver ion was weaker than that of α-linolenic acid containing isomer. The components were separated to the baseline with a resolution of 2.7.

The separation of triacylglycerols on silver-ion HPLC was also affected by the regiospecific distribution of fatty acids (*sn*-2 vs. *sn*-1/3) in a triacylglycerol molecule (Fig. 2, Table 2). Isomeric triacylglycerols differing in the position of unsaturated fatty acids were partially separated, whereas sat-

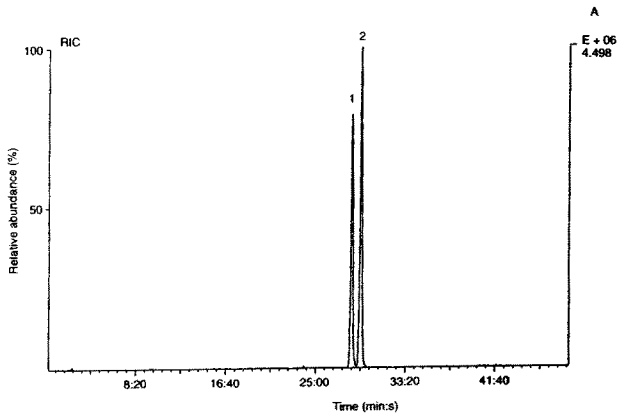


FIG. 1. Separation of 1,3-dioleoyl-2-γ-linolenoyl-*sn*-glycerol (peak number 1) and 1,3-dioleoyl-2-α-linolenoyl-*sn*-glycerol (peak number 2) by silver-ion high-performance liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry. The analytical conditions have been described in the Experimental Procedures section. RIC, reconstructed ion chromatogram.

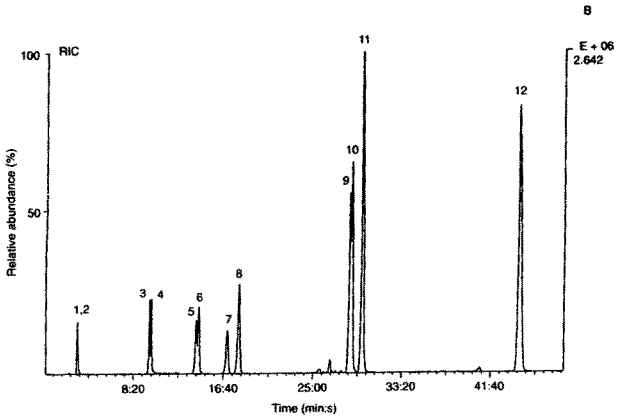


FIG. 2. Separation of triacylglycerols by silver-ion high-performance liquid chromatography according to the regiospecific distribution of fatty acids in the *sn*-2 and *sn*-1/3 positions. Components were detected by atmospheric pressure chemical ionization–mass spectrometry. Analytical conditions have been described in the Experimental Procedures section. Peak identifications: 1 = *rac*-1,2-distearoyl-3-palmitoyl-*sn*-glycerol, 2 = 1,3-distearoyl-2-palmitoyl-*sn*-glycerol, 3 = 1,3-dipalmitoyl-2-oleoyl-*sn*-glycerol, 4 = *rac*-1,2-dipalmitoyl-3-oleoyl-*sn*-glycerol, 5 = 1,3-dioleoyl-2-palmitoyl-*sn*-glycerol, 6 = *rac*-1,2-dioleoyl-3-palmitoyl-*sn*-glycerol, 7 = 1,3-distearoyl-2-linoleoyl-*sn*-glycerol, 8 = *rac*-1,2-dipalmitoyl-3-linoleoyl-*sn*-glycerol, 9 = 1,3-dioleoyl-2-γ-linolenoyl-*sn*-glycerol, 10 = *rac*-1,2-dioleoyl-3-γ-linolenoyl-*sn*-glycerol, 11 = trilinoleoylglycerol, 12 = trilinolenoylglycerol. See Figure 1 for abbreviation.

urated isomers were not separated at all due to the lack of interaction with silver ions. In general, the most unsaturated fatty acid moiety of a triacylglycerol esterified to the *sn*-1/3 position formed stronger interaction with silver ion than that esterified to the *sn*-2 position; for example, 1,3-dioleoyl-2-γ-linolenoyl-*sn*-glycerol eluted slightly earlier than *rac*-1,2-dioleoyl-3-γ-linolenoyl-*sn*-glycerol. With the chromatographic system used in the present study, *rac*-1,2-dioleoyl-3-palmitoyl-*sn*-glycerol had slightly stronger retention to the stationary phase than 1,3-dioleoyl-2-palmitoyl-*sn*-glycerol, thus, being an exception of the general rule. A reverse elution order of these two components or closely related ones has been achieved both by silver-ion TLC (30) and silver-ion HPLC (7,13,31). However, exceptions to the general elution order of isomeric triacylglycerols analyzed by silver-ion TLC has also been reported (30). Various eluent compositions used in separate studies may modify the stationary phase differently, thus, affecting the sample/stationary phase interaction and elution order of molecules. In addition to the regiospecific distribution of fatty acids, the separation also may be affected by differences in the chain length of the esterified fatty acyl residues, as was assumed from the good baseline separation (resolution 3.1) of 1,3-dioleoyl-2-linoleoyl-*sn*-glycerol and *rac*-1,2-dipalmitoyl-3-linoleoyl-*sn*-glycerol. Adlof (32) has recently reported the effect of chain length on the separation of triacylglycerols, diacylglycerol-monoacetates, monoacylglycerol-diacetates, and triacetin.

The regiospecific distribution of fatty acids also had an effect on the mass spectra of triacylglycerols obtained by APCI–MS (Table 2). The abundance of $[M - RCO_2]^+$ ion formed by a loss of a fatty acyl residue from the *sn*-2 position was less than that formed by a loss of a fatty acyl residue from the primary glycerol positions. As an example, the relative abundance of $[M - 16:0]^+$ ion in the APCI mass spectrum of 1,3-dioleoyl-2-palmitoyl-*sn*-glycerol was 8.5% of the abundance of the base peak, and in the mass spectrum of *rac*-1,2-dioleoyl-3-palmitoyl-*sn*-glycerol, it was 69.9%. This confirms the elution order of these regioisomeric triacylglycerols discussed above.

Silver-ion HPLC/APCI–MS of seed oils. Triacylglycerols of the seed oils studied were efficiently separated by silver-ion HPLC/APCI–MS as can be seen from the reconstructed ion chromatograms (RIC) (same as total ion chromatograms) shown in Figures 3 and 4. The analyses of triacylglycerols by silver-ion HPLC combined with ELSD produced very similar separations, differing mainly in the proportions of the separated fractions and, therefore, the ELSD chromatograms are not presented here. The identification of the eluted compo-

TABLE 2
Retention Properties and Mass Spectrometric Fragmentation of Triacylglycerols Analyzed by Silver-Ion HPLC/APCI–MS

Compound number	Triacylglycerol	t_r (min:s)	$[M + H]^+$ *m/z* (abundance)	$[M - R_1COO]^+$ *m/z* (abundance) [ion]	$[M - R_2COO]^+$ *m/z* (abundance) [ion]	Resolution (*R*)
1	*rac*-18:0-18:0-16:0	3:05	863.8 (n.d.)	579.5 (100.0) = $[M - 18:0]^+$	607.6 (56.4) = $[M - 16:0]^+$	$R_{1,2}$[a] = 0.00
2	*sn*-18:0-16:0-18:0[b]	3:05				
3	*sn*-16:0-18:1-16:0	9:49	833.8 (8.9)	551.5 (33.5) = $[M - 18:1]^+$	577.6 (100.0) = $[M - 16:0]^+$	$R_{3,4}$ = 0.99
4	*rac*-16:0-16:0-18:1	9:58	833.8 (16.2)	551.5 (88.7) = $[M - 18:1]^+$	577.6 (100.0) = $[M - 16:0]^+$	
5	*sn*-18:1-16:0-18:1	14:11	859.8 (20.7)	577.5 (100.0) = $[M - 18:1]^+$	603.6 (8.5) = $[M - 16:0]^+$	$R_{5,6}$ = 0.74
6	*rac*-18:1-18:1-16:0	14:24	859.8 (14.9)	577.5 (100.0) = $[M - 18:1]^+$	603.6 (69.9) = $[M - 16:0]^+$	
7	*sn*-18:0-18:2-18:0	17:01	887.8 (3.8)	603.5 (100.0) = $[M - 18:0]^+$	607.5 (37.5) = $[M - 18:2]^+$	$R_{7,8}$ = 3.09
8	*rac*-16:0-16:0-18:2	18:06	831.7 (5.4)	551.4 (100.0) = $[M - 18:2]^+$	575.5 (75.6) = $[M - 16:0]^+$	
9	*sn*-18:1-18:3n-6-18:1	28:26	881.8 (100.0)	599.5 (65.3) = $[M - 18:1]^+$	603.4 (59.0) = $[M - 18:3]^+$	$R_{9,10}$ = 0.57
10	*rac*-18:1-18:1-18:3n-6	28:39	881.8 (90.5)	599.5 (50.8) = $[M - 18:1]^+$	603.4 (100.0) = $[M - 18:3]^+$	
11	18:2-18:2-18:2	29:39	879.7 (100.0)	599.4 (51.5) = $[M - 18:2]^+$		
12	18:3n-3-18:3n-3-18:3n-3	44:20	873.7 (100.0)	595.3 (44.9) = $[M - 18:3]^+$		

[a]Subscripts refer to the compound numbers. [b]Co-eluted with the compound number 1.HPLC/APCI–MS, high-performance liquid chromatography/atmospheric pressure chemical ionization–mass spectrometry.

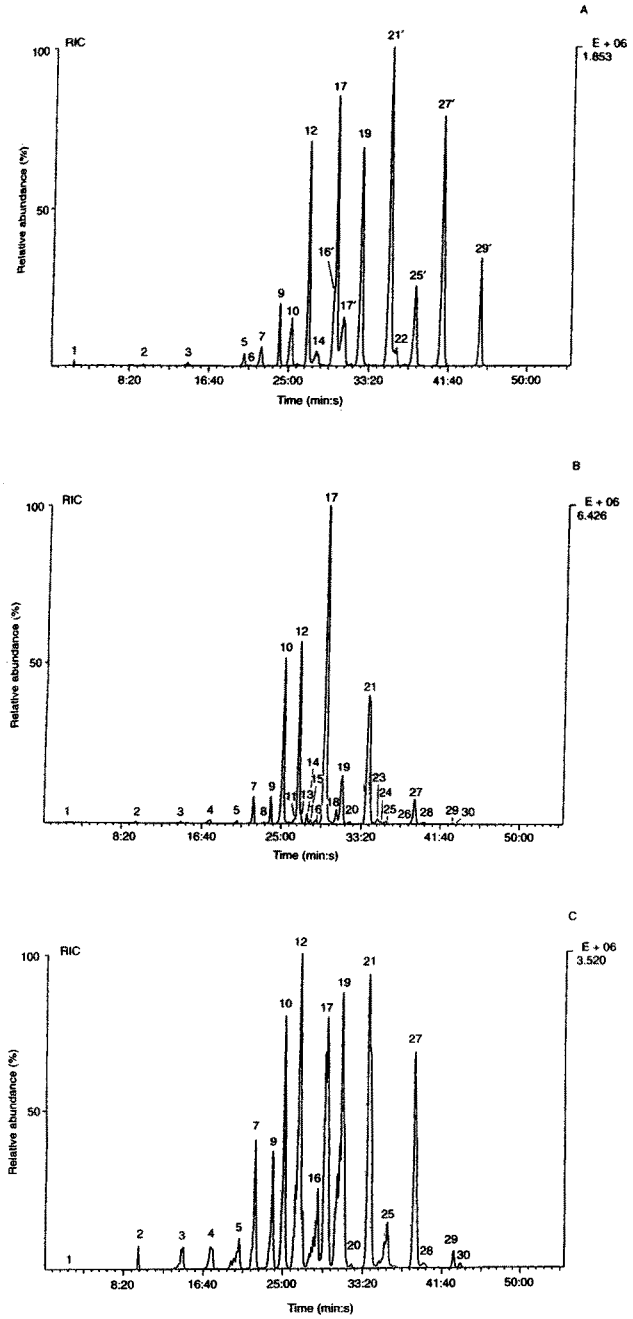


FIG. 3. RIC of the triacylglycerols of A) cloudberry seed oil, B) evening primrose oil, and C) borage oil achieved by silver-ion high-performance liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry. A seven-point smoothing has been applied to the chromatograms. The analytical details have been described in the Experimental Procedures section. Peak numbers refer to Table 3. See Figure 1 for abbreviation.

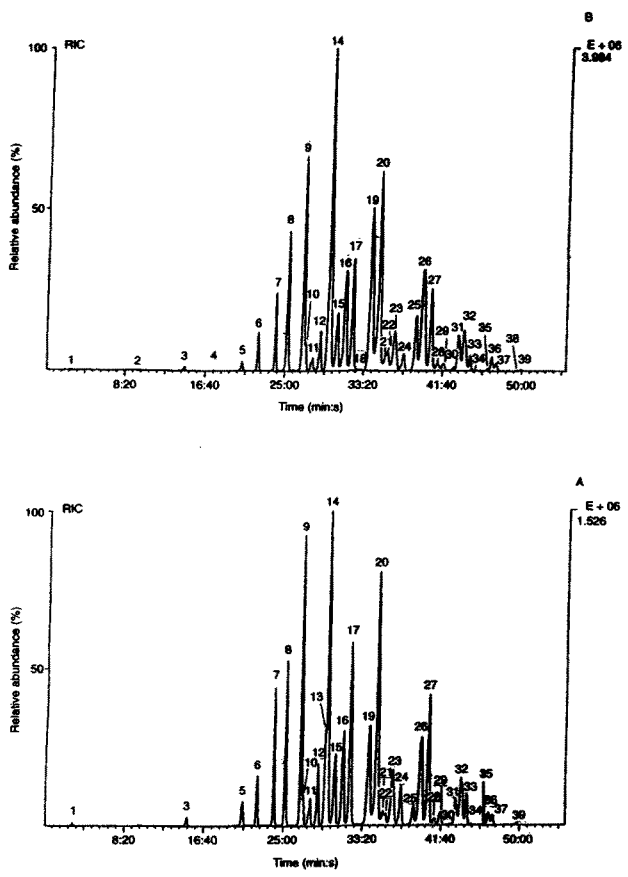


FIG. 4. RIC of the triacylglycerols of A) alpine currant seed oil, and B) black currant seed oil achieved by silver-ion high-performance liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry. A seven-point smoothing has been applied to the chromatograms. The analytical details have been described in the Experimental Procedures section. Peak numbers refer to Table 4. See Figure 1 for abbreviation.

nents and their proportions are presented in Table 3 and 4. Almost all the molecular species listed in the tables exhibited both $[M + H]^+$ and $[M - RCO_2]^+$ ions in the mass spectra. Triacylglycerols having together two or fewer double bonds in their acyl chains formed no or only a weak molecular ion and, thus, they were identified mainly according to the ions formed by the loss of one fatty acid moiety. None of the samples analyzed contained significant amounts of saturated triacylglycerols; thus, the first eluted peak without retention to the silver-ion column was possibly due to the presence of carotenoids, especially in cloudberry seed oil. The quantitative estimates of the separated triacylglycerol fractions were based on the peak areas in the RIC (APCI–MS) and in the ELSD chromatograms without corrections according to molecular response factors. In the present study, no attempt was made to estimate the relative amounts of different molecular species eluting in a single chromatographic peak. The proportions of triacylglycerols obtained by the two detection systems were not always in accordance, which was due to variable detector response of different triacylglycerol species. In the following, the analysis of each sample by silver-ion HPLC/APCI–MS will be discussed in more detail.

(i) Cloudberry seed oil. The elution order of the triacylglycerols of cloudberry seed oil, representing an oil rich in α-linolenic acid, was $SSM > SMM > MMM > SMD > MMD > SDD > MDD > SMT_\alpha > MMT_\alpha = DDD > SDT_\alpha > MDT_\alpha >$

TABLE 3
Identification of the Triacylglycerols of Cloudberry Seed Oil, Evening Primrose Oil, and Borage Oil by Silver-Ion HPLC/APCI–MS

		Cloudberry seed oil				Evening primrose oil				Borage oil			
Peak number	Abbreviation[a]	ACN:*n*[b]	Triacylglycerol[c]	MS[d] %	ELSD[e] %	ACN:*n*	Triacylglycerol	MS %	ELSD %	ACN:*n*	Triacylglycerol	MS %	ELSD %
1		Not triacylglycerols		0.1	[f]	Not triacylglycerols		<0.1	0.5	Not triacylglycerols		<0.1	n.d.
2	*SSM*	50:1	**16:0/16:0/18:1**[g]	<0.1	0.3	50:1	**16:0/16:0/18:1**	0.1	n.d.[h]	50:1	**16:0/16:0/18:1**	0.3	0.3
		52:1	16:0/18:0/18:1			52:1	16:0/18:0/18:1			52:1	16:0/18:0/18:1		
		54:1	18:0/18:0/18:1			54:1	18:0/18:0/18:1			54:1	18:0/18:0/18:1		
		56:1	18:0/20:0/18:1							56:1	18:0/20:0/18:1		
		58:1	18:0/22:0/18:1							58:1	18:0/22:0/18:1		
3	*SMM*	50:2	16:0/16:1/18:1	0.2	0.5	52:2	**16:0/18:1/18:1**	0.1	n.d.	52:2	**16:0/18:1/18:1**	1.0	0.7
		52:2	**16:0/18:1/18:1**			54:2	18:0/18:1/18:1			54:2	18:0/18:1/18:1 + 16:0/18:1/20:1		
		54:2	18:0/18:1/18:1							56:2	18:0/18:1/20:1 + 16:0/18:1/22:1		
		56:2	20:0/18:1/18:1							58:2	18:0/18:1/22:1 + 16:0/18:1/24:1		
		58:2	22:0/18:1/18:1										
4	SSD					50:2	**16:0/16:0/18:2**	0.5	n.d.	50:2	**16:0/16:0/18:2**	1.3	0.4
						52:2	16:0/18:0/18:2			52:2	16:0/18:0/18:2		
						54:2	18:0/18:0/18:2			54:2	18:0/18:0/18:2 + 16:0/20:0/18:2		
										56:2	18:0/20:0/18:2 + 16:0/22:0/18:2		
										58:2	18:0/22:0/18:2 + 16:0/24:0/18:2		
5	*MMM*	54:3	18:1/18:1/18:1	0.5	3.2	54:3	18:1/18:1/18:1	0.3	0.3	54:3	**18:1/18:1/18:1**	1.8	0.6
										56:3	18:1/18:1/20:1		
										58:3	18:1/18:1/22:1		
										60:3	18:1/18:1/24:1		
6	*MMM*	50:3	16:1/16:1/18:1	<0.1	n.d.								
7	*SMD*	52:3	**16:0/18:1/18:2**	1.1	0.8	52:3	**16:0/18:1/18:2**	2.1	1.3	52:3	**16:0/18:1/18:2**	4.2	8.1
		54:3	18:0/18:1/18:2			54:3	18:0/18:1/18:2			54:3	18:0/18:1/18:2 + 16:0/20:1/18:2		
		56:3	20:0/18:1/18:2			56:3	20:0/18:1/18:2			56:3	18:0/20:1/18:2 + 16:0/22:1/18:2		
		58:3	22:0/18:1/18:2							58:3	18:0/22:1/18:2 + 16:0/24:1/18:2		
										60:3	18:0/24:1/18:2		
8	*SDD*[j]					52:4	16:0/18:2/18:2[i]	0.1	n.d.				
9	*MMD*	54:4	**18:1/18:1/18:2**	2.2	4.5	54:4	**18:1/18:1/18:2**	1.5	0.7	54:4	**18:1/18:1/18:2**	3.5	5.5
		56:4	18:1/20:1/18:2			56:4	18:1/20:1/18:2			56:4	18:1/20:1/18:2		
										58:4	18:1/22:1/18:2 + 20:1/20:1/18:2		
										60:4	18:1/24:1/18:2 + 20:1/22:1/18:2		
10	*SDD*	52:4	**16:0/18:2/18:2**	3.3	1.4	52:4	**16:0/18:2/18:2**	13.7	10.5	50:4	16:0/16:2/18:2	8.6	8.2
		54:4	18:0/18:2/18:2			54:4	18:0/18:2/18:2			52:4	**16:0/18:2/18:2**		
		56:4	20:0/18:2/18:2			56:4	20:0/18:2/18:2			54:4	18:0/18:2/18:2		
		58:4	22:0/18:2/18:2							56:4	20:0/18:2/18:2		
		60:4	24:0/18:2/18:2							58:4	22:0/18:2/18:2		
11	SDT_{γ}[j]					52:5	16:0/18:2/18:3[j]	0.3	n.d.				
12	$MDD + SMT_{\gamma}$	54:5	**18:1/18:2/18:2**	8.8	14.3	54:5	18:1/18:2/18:2	13.7	15.3	52:4	16:0/18:1/18:3	16.1	23.7
		56:5	20:1/18:2/18:2							54:4	16:0/20:1/18:3		
										56:4	16:0/22:1/18:3		
										58:4	16:0/24:1/18:3		
										54:5	**18:1/18:2/18:2**		
										56:5	20:1/18:2/18:2		
										58:5	22:1/18:2/18:2		
										60:5	24:1/18:2/18:2		
13	SMT_{γ}					52:4	16:0/18:1/18:3	0.2	n.d.				
14	$SMT_{\alpha} + DDD$[j]	52:4	**16:0/18:1/18:3**	1.1	1.9	54:6	18:2/18:2/18:2[i]	0.6	n.d.				
		54:4	18:0/18:1/18:3										
		56:4	20:0/18:1/18:3										
15	MDT_{γ}[j]					54:6	18:1/18:2/18:3[i]	0.2	n.d.				

(continued on next page)

Peak number	Abbreviation[a]	Cloudberry seed oil ACN:n[b]	Cloudberry seed oil Triacylglycerol[c]	Cloudberry seed oil MS[d] %	Cloudberry seed oil ELSD[e] %	Evening primrose oil ACN:n	Evening primrose oil Triacylglycerol	Evening primrose oil MS %	Evening primrose oil ELSD %	Borage oil ACN:n	Borage oil Triacylglycerol	Borage oil MS %	Borage oil ELSD %
16	MMT_γ					54:5	18:1/18:1/18:3	0.3	n.d.	54:5	**18:1/18:1/18:3**	3.5	1.5
										56:5	18:1/20:1/18:3		
										58:5	18:1/22:1/18:3 + 20:1/20:1/18:3		
										60:5	18:1/24:1/18:3 + 20:1/22:1/18:3		
										62:5	20:1/24:1/18:3		
16′	MMT_α	54:5	**18:1/18:1/18:3**	3.3	n.d.								
		56:5	18:1/20:1/18:3										
17	$DDD + SDT_\gamma$	54:6	18:2/18:2/18:2	11.4	14.9	52:5	16:0/18:2/18:3	34.7	59.9	52:5	**16:0/18:2/18:3**	14.0	14.5
						54:6	**18:2/18:2/18:2**			54:6	18:2/18:2/18:2		
										54:5	18:0/18:2/18:3		
										56:5	20:0/18:2/18:3		
17′	SDT_α	52:5	**16:0/18:2/18:3**	3.6	1.5								
		54:5	18:0/18:2/18:3										
		56:5	20:0/18:2/18:3										
18	DDT_γ[i]					54:7	18:2/18:2/18:3[i]	1.0	n.d.				
19	MDT_γ					54:6	18:1/18:2/18:3	5.5	1.2	52:6	16:1/18:2/18:3	16.9	16.4
										54:6	**18:1/18:2/18:3**		
										56:6	20:1/18:2/18:3		
										58:6	22:1/18:2/18:3		
										60:6	24:1/18:2/18:3		
19′	MDT_α	54:6	**18:1/18:2/18:3**	13.7	17.4								
		56:6	20:1/18:2/18:3										
20	$DDT_\gamma + MDT_\alpha$[i]					54:7	**18:2/18:2/18:3**	0.2	n.d.	54:7	18:2/18:2/18:3	<0.1	n.d.
						54:6	18:1/18:2/18:3[i]			54:6	**18:1/18:2/18:3**		
21	$DDT_\gamma + ST_\gamma T_\gamma$					54:7	18:2/18:2/18:3	20.8	10.2	52:6	16:0/18:3/18:3	16.4	16.0
										54:7	**18:2/18:2/18:3**		
21′	DDT_α	54:7	**18:2/18:2/18:3**	21.1	27.7								
		56:7	18:2/20:2/18:3										
22	$ST_\alpha T_\alpha$	52:6	16:0/18:3/18:3	1.0	n.d.								
23	DDT_α[i]					54:7	18:2/18:2/18:3[i]	0.4	n.d.				
24	$DT_\gamma T_\gamma$					54:8	18:2/18:3/18:3	0.1	n.d.				
25	$MT_\gamma T_\gamma$					54:7	18:1/18:3/18:3	0.3	n.d.	52:7	16:1/18:3/18:3	2.3	0.4
										54:7	**18:1/18:3/18:3**		
										56:7	20:1/18:3/18:3		
										58:7	22:1/18:3/18:3		
										60:7	24:1/18:3/18:3		
25′	$MT_\alpha T_\alpha$	54:7	**18:1/18:3/18:3**	5.8	1.8								
		56:7	20:1/18:3/18:3										
26	$MDTe$					54:7	18:1/18:2/18:4						
27	$DT_\gamma T_\gamma$					54:8	18:2/18:3/18:3	2.9	0.2	54:8	18:2/18:3/18:3	9.4	3.7
27′	$DT_\alpha T_\alpha$	54:8	18:2/18:3/18:3	16.9	8.2								
28	$DDTe$					54:8	18:2/18:2/18:4	0.2	n.d.	54:8	18:2/18:2/18:4	0.1	n.d.
29	$T_\gamma T_\gamma T_\gamma$					54:9	18:3/18:3/18:3	0.1	n.d.	54:9	18:3/18:3/18:3	0.5	0.1
29′	$T_\alpha T_\alpha T_\alpha$	54:9	18:3/18:3/18:3	5.7	1.2								
30	$DT_\gamma Te$					54:9	18:2/18:3/18:4	<0.1	n.d.	54:9	18:2/18:3/18:4	0.1	n.d.

[a]Fatty acyl residues: S = saturated, M = monoenoic, D = dienoic, T_α = α-linolenic acid, T_γ = γ-linolenic acid, Te = tetraenoic fatty acid moiety. [b]ACN is the combined number of acyl carbons and n the combined number of double bonds in the acyl chains of a triacylglycerol. [c]The distribution of fatty acyl residues between *sn*-1, *sn*-2, and *sn*-3 positions has not been differentiated. [d]Proportions of triacylglycerols based on mass spectrometric (MS) detection. [e]Proportions of triacylglycerols based on evaporative light-scattering detection (ELSD). [f]The proportion was 23.4% of all peaks in ELSD chromatogram, %ELSD proportions presented here are calculated excluding the proportion of peak 1. [g]The triacylgycerol exhibiting the most abundant $[M + H]^+$ ion in the spectrum has been typed in bold font. [h]n.d. = Not detected or separated. [i]Tentative identification.

$DDT_\alpha \geq ST_\alpha T_\alpha > MT_\alpha T_\alpha > DT_\alpha T_\alpha > T_\alpha T_\alpha T_\alpha$, where S = saturated, M = monoenoic, and D = dienoic fatty acyl residue, and T_α = α-linolenoyl residue (Fig. 3A; Table 3). The elution order was similar to that reported earlier for linseed oil (6) with few exceptions. In the present study, one α-linolenoyl residue formed stronger interaction with silver ion than two linoleoyl residues, resulting in nearly baseline separations of *MDD* and SMT_α fractions, as well as *DDD* and SDT_α frac-

TABLE 4
Identification of the Triacylglycerols of Alpine Currant Seed Oil and Black Currant Seed Oil by Silver-Ion HPLC/APCI–MS

Peak number	Abbreviation[a]	Alpine currant seed oil ACN:*n*[b]	Alpine currant seed oil Triacylglycerol[c]	Alpine currant seed oil MS[d] (%)	Alpine currant seed oil ELSD[e] (%)	Black currant seed oil ACN:*n*	Black currant seed oil Triacylglycerol	Black currant seed oil MS (%)	Black currant seed oil ELSD (%)
1		Something other than triacylglycerols		0.1	4.2	Something other than triacylglycerols		<0.1	0.6
2	*SSM*					52:1 54:1	16:0/18:0/18:1 18:0/18:0/18:1	<0.1	0.1
3	*SMM*	52:2 54:2	**16:0/18:1/18:1**[f] 18:0/18:1/18:1	0.3	<0.1	52:2 54:2	**16:0/18:1/18:1** 18:0/18:1/18:1	0.1	0.1
4	*SSD*					52:2	16:0/18:0/18:2	<0.1	n.d.[g]
5	*MMM*	54:3	18:1/18:1/18:1	1.0	0.2	54:3	18:1/18:1/18:1	0.4	0.1
6	*SMD*	52:3 54:3	**16:0/18:1/18:2** 18:0/18:1/18:2	1.6	2.2	52:3 54:3	**16:0/18:1/18:2** 18:0/18:1/18:2	1.3	1.7
7	*MMD*	54:4	18:1/18:1/18:2	3.2	7.1	54:4 56:4	**18:1/18:1/18:2** 18:1/20:1/18:2	2.1	3.7
8	*SDD*	52:4 54:4	**16:0/18:2/18:2** 18:0/18:2/18:2	5.1	4.0	52:4 54:4	**16:0/18:2/18:2** 18:0/18:2/18:2	5.1	5.3
9	*MDD*	54:5	18:1/18:2/18:2	9.2	17.5	54:5 56:5	**18:1/18:2/18:2** 20:1/18:2/18:2	8.3	15.0
10	SMT_γ	52:4	16:0/18:1/18:3	0.6	n.d.	52:4	16:0/18:1/18:3	0.6	nd
11	SMT_α	52:4 54:4	**16:0/18:1/18:3** 18:0/18:1/18:3	1.0	0.4	52:4 54:4	**16:0/18:1/18:3** 18:0/18:1/18:3	0.5	0.2
12	MMT_γ	54:5	18:1/18:1/18:3	2.0	0.9	54:5 56:5	**18:1/18:1/18:3** 18:1/20:1/18:3	1.6	0.6
13	MMT_α	54:5	18:1/18:1/18:3	2.7	n.d.				
14	SDT_γ + *DDD* + MMT_α	52:5 54:6	16:0/18:2/18:3 **18:2/18:2/18:2**	11.9	17.2	52:5 54:5 54:6	16:0/18:2/18:3 18:1/18:1/18:3 **18:2/18:2/18:2**	15.9	25.4
15	SDT_α	52:5 54:5	**16:0/18:2/18:3** 18:0/18:2/18:3	3.4	2.2	52:5 54:5	**16:0/18:2/18:3** 18:0/18:2/18:3	2.8	1.3
16	MDT_γ	54:6	18:1/18:2/18:3	4.6	3.8	54:6 56:6	**18:1/18:2/18:3** 20:1/18:2/18:3	6.1	5.6
17	MDT_α	54:6	18:1/18:2/18:3	7.8	12.1	54:6 56:6	**18:1/18:2/18:3** 20:1/18:2/18:3	5.2	4.7
18	DDT_γ					54:7	18:2/18:2/18:3	<0.1	n.d.
19	DDT_γ + $ST_\gamma T_\gamma$	52:6 54:7	16:0/18:3/18:3 **18:2/18:2/18:3**	6.1	4.6	52:6 54:7	16:0/18:3/18:3 **18:2/18:2/18:3**	11.6	13.6
20	DDT_α	54:7	18:2/18:2/18:3	12.3	14.7	54:7	18:2/18:2/18:3	11.1	12.9
21	$ST_\alpha T_\alpha$ + $MT_\gamma T_\gamma$	52:6	16:0/18:3/18:3	0.3	n.d.	52:6 56:7	**16:0/18:3/18:3** 20:1/18:3/18:3	0.4	0.1
22	$MT_\gamma T_\gamma$	54:7	18:1/18:3/18:3	0.3	0.4	54:7	18:1/18:3/18:3	0.5	0.2
23	$MT_\gamma T_\alpha$ + *MDTe*	54:7	18:1/18:3/18:3 + 18:1/18:2/18:4	3.3	1.5	54:7	18:1/18:2/18:4 + 18:1/18:3/18:3	2.6	0.9
24	$MT_\alpha T_\alpha$	54:7	18:1/18:3/18:3	2.0	0.6	54:7	18:1/18:3/18:3	0.8	0.1
25	$DT_\gamma T_\gamma$	54:8	18:2/18:3/18:3	1.0	n.d.	54:8	18:2/18:3/18:3	3.1	1.0
26	$DT_\gamma T_\alpha$ + *DDTe*	54:8	18:2/18:3/18:3 + 18:2/18:2/18:4	6.3	2.4	54:8	18:2/18:3/18:3 + 18:2/18:2/18:4	8.3	4.0
27	$DT_\alpha T_\alpha$	54:8	18:2/18:3/18:3	6.1	2.5	54:8	18:2/18:3/18:3	4.1	1.2
28	$MT_\gamma Te$	54:8	18:1/18:3/18:4	0.2	0.1	54:8	18:1/18:3/18:4	0.2	<0.1
29	$MT_\alpha Te$	54:8	18:1/18:3/18:4	0.6	0.2	54:8	18:1/18:3/18:4	0.3	<0.1
30	$T_\gamma T_\gamma T_\gamma$	54:9	18:3/18:3/18:3	<0.1	n.d.	54:9	18:3/18:3/18:3	0.1	n.d.
31	$T_\gamma T_\gamma T_\alpha$ + $DT_\gamma Te$	54:9	18:3/18:3/18:3 + 18:2/18:3/18:4	1.5	0.3	54:9	18:2/18:3/18:4 + 18:3/18:3/18:3	2.2	0.6
32	$T_\gamma T_\alpha T_\alpha$ + $DT_\alpha Te$	54:9	18:2/18:3/18:4	3.2	0.9	54:9	18:2/18:3/18:4 + 18:3/18:3/18:3	2.7	0.6
33	$T_\alpha T_\alpha T_\alpha$	54:9	18:3/18:3/18:3	1.3	0.2	54:9	18:3/18:3/18:3	0.6	<0.1
34	*MTeTe*	54:9	18:1/18:4/18:4	<0.1	n.d.	54:9	18:1/18:4/18:4	<0.1	n.d.

(continued on next page)

Peak number	Abbreviation[a]	Alpine currant seed oil ACN:n[b]	Triacylglycerol[c]	MS[d] (%)	ELSD[e] (%)	Black currant seed oil ACN:n	Triacylglycerol	MS (%)	ELSD (%)
35	$T_\gamma T_\gamma Te$	54:10	18:3/18:3/18:4	<0.1	n.d.	54:10	18:3/18:3/18:4	0.1	n.d.
36	$T_\gamma T_\alpha Te$	54:10	18:3/18:3/18:4	0.6	0.2	54:10	18:3/18:3/18:4	0.7	0.1
37	$T_\alpha T_\alpha Te$	54:10	18:3/18:3/18:4	0.5	0.1	54:10	18:3/18:3/18:4	0.4	<0.1
38	$T_\gamma TeTe$					54:11	18:3/18:4/18:4	<0.1	n.d.
39	$T_\alpha TeTe$	54:11	18:3/18:4/18:4	0.1	n.d.	54:11	18:3/18:4/18:4	0.1	n.d.

[a]Fatty acyl residues: *S* = saturated, *M* = monoenoic, *D* = dienoic, T_α = α-linolenic acid, T_γ = γ-linolenic acid, *Te* = tetraenoic fatty acid moiety.
[b]ACN is the combined number of acyl carbons and *n* the combined number of double bonds in the acyl chains of a triacylglycerol.
[c]The distribution of fatty acyl residues between *sn*-1, *sn*-2, and *sn*-3 positions has not been differentiated.
[d]Proportions of triacylglycerols based on mass spectrometric (MS) detection.
[e]Proportions of triacylgycerols based on evaporative light scattering detection (ELSD).
[f]The triacylgycerol exhibiting the most abundant $[M + H]^+$ ion in the spectrum has been typed in bold font.
[g]n.d. = Not detected or separated.

tions. Furthermore, $ST_\alpha T_\alpha$ eluted slightly after DDT_α as a shoulder peak, and MMT_α and *DDD* eluted in the same peak. In the case of linseed oil (6), *MDD* co-eluted with SMT_α, and *DDD* co-eluted with SDT_α whereas MMT_α and *DDD* were well separated. Such differences in the elution order of triacylglycerols may be caused by minor differences in the column performance or in the analytical conditions. The most abundant triacylglycerols of cloudberry seed oil, separated by silver-ion HPLC, represented *MDD*, *DDD*, MDT_α, DDT_α, and $DT_\alpha T_\alpha$ fractions. The most abundant molecular species of these fractions were 18:1/18:2/18:2, 18:2/18:2/18:2, 18:1/18:2/18:3n-3, 18:2/18:2/18:3n-3, and 18:2/18:3n-3/18:3n-3, respectively (distribution of fatty acids between *sn*-1, *sn*-2, and *sn*-3 positions is not differentiated). Overall, 43 molecular species of triacylglycerols were identified from cloudberry seed oil.

(ii) Evening primrose oil. The general elution order of the triacylglycerols of evening primrose oil (Fig. 3B; Table 3) was much the same as that for cloudberry seed oil. In addition, evening primrose contained a small *SSD* fraction which eluted between *SMM* and *MMM* fractions. Differences were observed in the retention behavior of triacylglycerols containing trienoic fatty acyl residues. The presence of γ-linolenic acid resulted in co-elution of *MDD* and SMT_γ fractions, and *DDD* with SDT_γ, where T_γ = γ-linolenoyl residue. Furthermore, the MMT_γ fraction eluted before *DDD* + SDT_γ fraction. Comparison of the retention times of the triacylglycerols of evening primrose oil with those of cloudberry seed oil showed that SMT_γ eluted before SMT_α, MMT_γ before MMT_α, SDT_γ before SDT_α, MDT_γ before MDT_α, DDT_γ before DDT_α, $MT_\gamma T_\gamma$ before $MT_\alpha T_\alpha$, $DT_\gamma T_\gamma$ before $DT_\alpha T_\alpha$, and $T_\gamma T_\gamma T_\gamma$ before $T_\alpha T_\alpha T_\alpha$. Thus, triacylglycerols containing γ-linolenoyl residues formed weaker interactions with silver ions than molecules containing α-linolenoyl residues. The general elution profile of the triacylglycerols of evening primrose oil was very similar to that reported by Christie (19). APCI–MS detection allowed achievement of information on the molecular composition of the minor constituents as well; therefore, more components were resolved and identified in the present study (39 molecular species). Identification of some minor fractions was only tentative, because a fraction yielding a similar mass spectrum was identified elsewhere in the chromatogram. Differences in the regiospecific distribution of fatty acyl residues in triacylglycerols as well as the presence of small amount of α-linolenic acid in evening primrose oil may partially explain this observation. In general, molecular species of triacylglycerols containing dienoic fatty acyl residues were typical constituents of evening primrose oil. The most abundant fractions separated by silver-ion HPLC were *SDD*, *MDD*, *DDD* + SDT_γ, and DDT_γ consisting of the most abundant triacylglycerols 16:0/18:2/18:2, 18:1/18:2/18:2, 18:2/18:2/18:2, and 18:2/18:2/18:3n-6, respectively.

(iii) Borage oil. The analysis of the triacylglycerols of borage oil by silver-ion HPLC/APCI–MS (Fig. 3C; Table 3) resulted in a more complex separation compared with evening primrose oil. The main elution order of the triacylglycerols of both γ-linolenic acid oils was similar. An additional feature of borage oil was the presence of $ST_\gamma T_\gamma$ which eluted in the same peak as DDT_γ. Both borage oil and evening primrose oil contained small amounts of triacylglycerols with tetraenoic fatty acyl residues (*Te*), such as *DDTe* and $DT_\gamma Te$. Several of the peaks in the reconstructed ion chromatogram of borage oil showed a deformed peak shape. This was actually caused by partial separation of molecular species within a single peak representing equally unsaturated molecules. In general, the molecules eluted in the descending order of molecular weight, e.g., the elution order of triacylglycerols within the *MMM* fraction (peak number 5) was 60:3 ($[M + H]^+$ ion, *m/z* 969.9) followed by 58:3 (*m/z* 941.9), 56:3 (*m/z* 913.8), and finally 54:3 (*m/z* 885.8). Moreover, the elution order of molecular species was 18:1/18:1/24:1 > 18:1/18:1/22:1 > 18:1/18:1/20:1 > 18:1/18:1/18:1. Similar separations were determined within several other peaks containing saturated and/or monoenoic fatty acid moieties, the chain length of which varied from C_{16} to C_{24} in borage oil. The same phenomenon was also observed with other oils, but to lesser extent. Thus, the separation of triacylglycerols by silver-ion

HPLC is also affected by the chain length of the fatty acyl residues. Adlof (32) has suggested recently that this kind of separation may be due to substrate interaction with the free silanol groups or with the phenylsulphonic acid groups of the stationary phase. The most abundant triacylglycerol fractions of borage oil separated by silver-ion HPLC were *MDD* + SMT_γ, *DDD* + SDT_γ, MDT_γ, and $ST_\gamma T_\gamma$ + DDT_γ. The most abundant molecular species of these fractions were 18:1/18:2/18:2, 16:0/18:2/18:3n-6, 18:1/18:2/18:3n-6, and 18:2/18:2/18:3n-6, respectively. In this study, 79 molecular species of triacylglycerols were identified from borage oil, which shows the high complexity of the sample.

(iv) Alpine currant and black currant seed oils. The RIC of the triacylglycerols of both alpine currant and black currant seed oil (Fig. 4), achieved by silver-ion HPLC/APCI–MS, were more complicated than those discussed above. The triacylglycerols of the currants were separated into 39 peaks, representing molecules from *SSM* (one double bond) to *TTeTe* (11 double bonds). The identification of the molecular species and their proportions are presented in Table 4. Altogether 44 molecular species of triacylglycerols were identified from alpine currant, and 56 from black currant seed oil. The more complex composition of black currant was mainly due to the presence of C_{20} fatty acids in the oil. The elution order of the less unsaturated triacylglycerols of the currants was similar to that achieved with α-linolenic acid and γ-linolenic acid containing oils, i.e., molecules eluted in the order of *SSM* > *SMM* > *SSD* > *MMM* > *SMD* > *MMD* > *SDD* > *MDD*. Both isomers of triacylglycerols containing either one α-linolenoyl or one γ-linolenoyl residue were separated from alpine currant and black currant seed oils. The isomer pairs of SMT_γ–SMT_α, MMT_γ–MMT_α, SDT_γ–SDT_α, MDT_γ–MDT_α, DDT_γ–DDT_α, $MT_\gamma Te$–$MT_\alpha Te$, $DT_\gamma Te$–$DT_\alpha Te$, and $T_\gamma TeTe$–$T_\alpha TeTe$ were well separated so that the molecule containing γ-isomer had a shorter retention time compared with the molecule containing α-isomer. Triacylglycerols with SDT_γ, *DDD*, and MMT_α structures eluted in the same peak which was in accordance with the results achieved with cloudberry, evening primrose, and borage oils. Molecules containing two linolenoyl residues were separated up to three isomers, which eluted in the order of $XT_\gamma T_\gamma$ followed by $XT_\gamma T_\alpha$ followed by $XT_\alpha T_\alpha$, where *X* is a fatty acyl residue different from a linolenoyl residue. The elution order of isomers was $ST_\gamma T_\gamma$ > $ST_\alpha T_\alpha$, $MT_\gamma T_\gamma$ > $MT_\gamma T_\alpha$ > $MT_\alpha T_\alpha$, $DT_\gamma T_\gamma$ > $DT_\gamma T_\alpha$ > $DT_\alpha T_\alpha$, and $T_\gamma T_\gamma Te$ > $T_\gamma T_\alpha Te$ > $T_\gamma T_\alpha Te$. Trilinolenoylglycerols were separated into four isomers which eluted in the order of $T_\gamma T_\gamma T_\gamma$ followed by $T_\gamma T_\gamma T_\alpha$, $T_\gamma T_\alpha T_\alpha$, and finally $T_\alpha T_\alpha T_\alpha$.

Triacylglycerols of both currants consisted mainly of molecules with 52 or 54 acyl carbons. In addition, triacylglycerols with 56 acyl carbons were determined in black currant seed oil, which was in accordance with the determined fatty acid composition (approximately 1 mol% C_{20} acids; Table 1) and other studies (25). The elution profiles of triacylglycerols of black currant and alpine currant seed oils achieved by silver-ion HPLC were very similar as were the molecular species compositions of the separated peaks. However, differences were determined in the relative amounts of triacylglycerol fractions (Table 4). The most abundant triacylglycerols of alpine currant eluted in the fractions representing *MDD*, SDT_γ + *DDD*, MDT_α, and DDT_α molecules. The most abundant molecular species of these fractions were 18:1/18:2/18:2, 18:2/18:2/18:2, 18:1/18:2/18:3n-3, 18:2/18:2/18:3n-3, respectively. The main fractions of black currant seed oil were *MDD*, SDT_γ + *DDD* + MMT_α, DDT_γ + $ST_\alpha T_\gamma$, and DDT_α, and the most abundant molecular species in these fractions were 18:1/18:2/18:2, 18:2/18:2/18:2, 18:2/18:2/18:3n-6, and 18:2/18:2/18:3n-3, respectively. Differences were observed in the proportions of molecules containing α- and/or γ-linolenoyl residues within equally unsaturated triacylglycerols. Alpine currant contained substantially more 18:1/18:2/18:3n-3 (MDT_α, peak number 17) compared with 18:1/18:2/18:3n-6 (MDT_γ, peak number 16), whereas the proportions of these molecular species in black currant seed oil were nearly equal. Similarly, the relative amount of 18:2/18:2/18:3n-6 + 16:0/18:3n-6/18:3n-6 (DDT_γ + $ST_\gamma T_\gamma$, peak number 19) in alpine currant was clearly less than that of 18:2/18:2/18:3n-3 (DDT_α, peak number 20), whereas the corresponding proportions were nearly the same in black currant seed oil. Such differences were expected: the ratio of the proportions of γ-linolenic acid to α-linolenic acid was ~1:1 in black currant and ~1:2 in alpine currant seed oil. Among *DTT* + *DDTe*-molecules, alpine currant contained equal proportions of 18:2/18:3n-6/18:3n-3 + 18:2/18:2/18:4 (peak number 26) and 18:2/18:3n-3/18:3n-3 (peak number 27), whereas the relative amount of 18:2/18:3n-6/18:3n-6 (peak number 25) was substantially low. The corresponding triacylglycerols of black currant seed oil were richer in γ-linolenic acid containing molecules, i.e., 8% $DT_\gamma T_\alpha$ + *DDTe*, 3% $DT_\gamma T_\gamma$ and 4% $DT_\alpha T_\alpha$ according to quantitative estimates produced by APCI–MS.

DISCUSSION

The composition and structure of oils containing γ-linolenic acid are of increasing interest due to their possible positive physiological properties, such as in the treatment of atopic eczema and diabetic neuropathy, and revealing breast pain and premenstrual syndrome (33). The physiological effects of γ-linolenic acid oils have mostly been studied with evening primrose oil and borage oil. The properties of currant seed oils have not been studied intensively, although currants contain significant amounts of γ-linolenic acid and also stearidonic acid, both of which are Δ-6-desaturation products. The medical properties of these oils are due to biochemical molecular reactions, and thus it is important to achieve information on the molecular composition of triacylglycerols. The chromatographic separation of triacylglycerols containing α- and/or γ-linolenic acid moieties is essential, because triacylglycerols containing these fatty acids cannot be distinguished as such by MS detection. In the present study, silver-ion HPLC has been shown to be an efficient method for the sepa-

ration of α- and γ-linolenic acid containing triacylglycerols in complex mixtures. Furthermore, molecular species containing stearidonic acid were determined. The on-line detection of the separated compounds by APCI–MS made it possible to achieve information both on the molecular weight and the molecular association of fatty acids of the triacylglycerol molecular species. The *m/z* values of $[M + H]^+$ ions define unambiguously the number of acyl carbons and double bonds in the molecule. The identification of relatively saturated triacylglycerols may be problematic, because they do not yield any or only a weak $[M + H]^+$ ion. Therefore, the identification of, for example, milk-fat triacylglycerols may be more difficult, and additional information, such as retention time data, will be required. The mass spectra of triacylglycerols achieved by APCI–MS are affected by the structure of the molecule, e.g., the degree of unsaturation and the regiospecific distribution of fatty acids between the *sn*-2 and *sn*-1/3 positions, as well as by the instrumental parameters of the mass spectrometer, such as capillary and vaporizer temperatures. At the present stage of the method, no attempt was made for distinction of regioisomeric triacylglycerols in complex mixtures, although differences were measured in the relative abundances of $[M - RCO_2]^+$ ions formed by a loss of a fatty acyl residue from the *sn*-2 position and the *sn*-1/3 positions of triacylglycerol reference components. The regiospecific analysis of triacylglycerols would require extensive optimization and calibration of the system. The silver-loaded cation-exchange column showed potential to separate, at least partially, unsaturated regioisomeric triacylglycerols by using a ternary solvent gradient. Similar results have been reported earlier using an isocratic eluent system consisting of acetonitrile and hexane (31). It may be possible to separate regioisomers of relatively simple triacylglycerol mixtures with an optimized silver-ion HPLC analysis as reported elsewhere (7). However, the samples of the present study, especially currants, seem to be far too complex for such separation. The quantitative estimates presented in this study were based on uncalibrated systems, i.e., no corrections to the raw data were done according to different response factors of molecular species. The quantitative analysis of triacylglycerols requires to be thoroughly studied. The presence of several molecular species within a single chromatographic peak makes it difficult to quantitate single triacylglycerols. The molecular weights of components eluting in the same peak in silver-ion HPLC may be differentiated according to their $[M + H]^+$; however, a single $[M - RCO_2]^+$ ion may be a fragment formed by a loss of one fatty acyl residue from several molecules. It may be possible to modify the elution system and ionization conditions so that triacylglycerols would yield only abundant ions consisting of the whole molecule without fragmentation. Unfortunately, in such a situation the information on molecular association of fatty acids is lost. Although quantitation may cause difficulties, the on-line detection of triacylglycerols by APCI–MS after separation by silver-ion HPLC provides valuable information for structure elucidation. Mass spectra yield information on both the molecular weight and molecular association of fatty acids of triacylglycerols. Similar data cannot be achieved by GC analysis of fatty acid methyl esters of the collected fractions separated by silver-ion HPLC.

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Availability of Arachidonic Acid in Major Phospholipids of Mucosa and the Stomach Wall of Rats

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ABSTRACT: The purpose of this study was to examine the availability of arachidonic acid in phosphatdidylcholine (PC) and phosphatidylethanolamine (PE) of mucosa and the stomach wall of adult male Wistar rats fed a standard diet. There were significant differences in the fatty acid composition of PC and PE between various parts of the stomach. The mucosa had the lowest level of arachidonic acid (20:4n-6) in PC, followed by the corresponding stomach wall or glandular part, whereas the forestomach or the upper part had the highest level of 20:4n-6. The level of 20:4n-6 in PE was identical in gastric mucosa and the stomach wall of both the lower and upper parts. The levels of 18:1n-9 and 18:2n-6 were significantly higher in both PC and PE of mucosa than in the stomach wall. The levels of 16:0 and 18:0 in PC were lower in mucosa than in either forestomach or the glandular part. In mucosal PE, the levels of 18:0 and 22:6n-3 were lower than in forestomach and glandular part. The glandular part had significantly higher level of 16:0 and lower level of 20:4n-6 in PC compared to the forestomach. Conclusions: The arachidonic acid level of PC was significantly different in various parts of the rat stomach, with the lowest level in mucosa, whereas the level of 20:4n-6 in PE was identical in mucosa and stomach wall. These phospholipids had higher levels of saturated fatty acids in the stomach wall than in gastric mucosa.
Lipids 31, 1323–1325 (1996).

The rat stomach consists of three main parts, the forestomach or the upper part, and the glandular or lower part which also contains the mucosa. The upper and the lower parts of the stomach have different structures, and their roles in digestion are different since the lower part contains mucosa but not the upper part. In the forestomach above the limiting ridge, the epithelium is squamous, whereas below the limiting ridge (the body and antrum) it is glandular (1). On the luminal surface of the stomach there is a layer of surface-active phospholipids that render the mucosal surface hydrophobic and resistant to damage induced by luminal acid (2,3). Ulcerogenic substances such as aspirin cause a dissipation of this layer, leading to back-diffusion of acid and mucosal necrosis (4).

The discovery of an association between infection with *Helicobacter pylori* and peptic ulcer disease has stimulated further evaluation of mucosal defense (2). *Helicobacter pylori* release phospholipases and ammonia which have the capacity to attenuate the phospholipid-dependent hydrophobic lining of the stomach and reduce mucosal defenses. The majority of people infected with this bacterium do not, however, develop ulcers, suggesting that differences in mucosal response to infection determine whether or not an ulcer develops (5).

The susceptibility to gastric erosions or ulceration differs in different parts of the rat stomach, with the mucosa being most sensitive to damage. The purpose of this study was to compare the fatty acid composition of major phospholipids, phosphatidylcholine (PC), and phosphatidylethanolamine (PE), in different parts of the stomach. The availability of arachidonic acid was of particular interest since arachidonic acid is the precursor of prostaglandins which have been shown to have the ability to increase the resistance of gastric mucosa to damage (6).

EXPERIMENTAL PROCEDURES

Animals. Male Wistar rats were fed a standard diet (rat and mouse maintenance diet no. 1; Special Diets Services Ltd., Essex, United Kingdom). The diet contained 18.1% of 16:0, 1.6% of 18:0, 13.9% of 18:1n-9, 56.5% of 18:2n-6, and 6.5% of 18:3n-3. Other fatty acids were 0.5% or less of the total fatty acids. The fat content was 2.0% in the diet. The rats were housed in cages, 3–4 rats per cage, and maintained on 12/12 h light–dark cycle with free access to water and food. At the age of 10 mon, the rats were anesthetized with CO_2 and killed by decapitation. The rats were fasted for 18 h but had free access to water before decapitation. The stomachs were removed, opened along the greater curvature, and thoroughly washed with saline.

Lipid extraction. Gastric mucosa from three stomachs was pooled for each experiment. The lower glandular part (antrum and body) was gently scraped, and the mucosal scrapings immediately suspended in 100 mL of a mixture of chloroform/methanol (2:1, vol/vol). Each mucosal fraction was extracted in chloroform/methanol (2:1, vol/vol) for 8 h at room temperature. The solvent fraction was collected, and the precipitate was re-extracted in chloroform/methanol (1:2, vol/vol) for 8 h at room temperature. The procedure was then repeated with chloroform/methanol (1:1, vol/vol). The sol-

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Abbreviations: PC, phosphatdidylcholine; PE, phosphatidylethanolamine.

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vent fractions were combined and evaporated in a rotary evaporator.

Lipid analyses. Parts from three mucosally denuded stomachs were used for each analysis. The upper part of the stomach, the forestomach, was cut from the glandular part along the limiting ridge, and each part was analyzed separately, cut into small pieces in 76 mL of methanol/chloroform/water (2:1:0.8, by vol) (7) and homogenized in a Polytron PTA 10-35 homogenizer (Kinematica, Luzern, Switzerland) for 4 × 10 s. After filtration, a biphasic system was produced by dilution with one volume of chloroform and 0.73% NaCl solution. The lower layer was withdrawn and evaporated in a rotary evaporator.

The lipid extracts from mucosa and the two mucosally denuded stomach parts were dissolved in a small volume of chloroform and separated on silicic acid columns (1.5 × 30 cm) into three major lipid fractions: neutral lipids, eluted with chloroform; glycolipids, eluted with acetone/methanol (8:2, vol/vol); and phospholipids, eluted with methanol. This report describes only the study of the major phospholipids, PC and PE.

The phospholipids contained in the methanol fraction were separated by thin-layer chromatography on prewashed and activated silica gel 60 plates (Merck, Darmstadt, Germany). The developing solvent system consisted of chloroform/ethanol/triethylamine/water (30:34:35:8, by vol). After briefly drying the plates with nitrogen, they were sprayed with 0.01% Rhodamin 6G (Sigma, St. Louis, MO) in water and inspected under ultraviolet light. The phospholipids were identified by comparison with authentic standards and PC and PE bands were then scraped from the plates. The phospholipid fatty acids were methylated with the method of Morrison and Smith (8). Fatty acid methyl esters were analyzed in a Perkin-Elmer 8410 gas chromatograph (Norwalk, CT) equipped with capillary column type DB-255 (30 m × 0.25 mm). The gas chromatograph was fitted with a Perkin-Elmer AS 8300 autosampler. The carrier gas was hydrogen at 1.5 mL/min, and the injector temperature was set at 265°C. The oven temperature was programmed at 3°C/min from 180–210°C and then 210°C steady temperature for 15 min. The detector temperature was set at 265°C (9).

The statistical tests used in the analyses of data were one-way analysis of variance. Data are expressed as mean ± SE for five preparations where mucosa from three rat stomachs were pooled per preparation.

RESULTS

The fatty acid composition of PC and PE in the mucosa and mucosally denuded glandular part and forestomach are shown in Tables 1 and 2. There were marked differences in the fatty acid composition of PC between various parts of the stomach. The mucosa had significantly lower levels of 20:4n-6, 16:0, and 18:0 but higher levels of 18:1n-9 and 18:2n-6 than the denuded gastric wall, both forestomach and the glandular part. There were also significant differences in fatty acid composition of PC between forestomach and glandular parts of the stomach, the level of 16:0 was higher in PC of the glandular part than in the forestomach whereas 20:4n-6 was lower in the glandular part (Table 1).

TABLE 1
Fatty Acid Composition of Phosphatidylcholine for All Stomach Parts of Animals Fed Standard Diet[a]

Fatty acid	Mucosa	Glandular part	Forestomach
14:0	—	0.70 ± 0.18	0.65 ± 0.16
16:0	28.09 ± 0.46	42.45 ± 0.18a	35.40 ± 0.50a,b
16:1	2.87 ± 0.18	1.80 ± 0.07a	1.99 ± 0.04a
18:0	6.10 ± 0.27	10.06 ± 0.67a	12.05 ± 0.29a,b
18:1n-9	18.24 ± 0.89	8.57 ± 0.13a	9.65 ± 0.12a
18:1n-7	4.25 ± 0.16	6.36 ± 0.06a	4.66 ± 0.07b
18:2n-6	24.32 ± 0.77	8.45 ± 0.41a	9.69 ± 0.25a
20:4n-6	11.76 ± 0.53	15.39 ± 0.45a	20.82 ± 0.39a,b
22:0	0.27 ± 0.27	1.15 ± 0.18a	0.71 ± 0.18
22:6n-3	0.31 ± 0.31	0.52 ± 0.14	0.52 ± 0.21
Others[b]	3.70 ± 1.04	4.55 ± 0.69	3.86 ± 0.64

[a]Values are the means ± SE of five preparations. Three stomachs were pooled per preparation. a, $P < 0.05$ vs. mucosa; b, $P < 0.05$ vs. glandular part.
[b]Fatty acids which are below 0.3% of total fatty acids.

The level of 20:4n-6 in PE was, on the other hand, identical in mucosa and the stomach wall. There were significant differences in the fatty acid profile of PE between various parts of the stomach (Table 2). In the mucosa, the levels of 18:1n-9 and 18:2n-6 were significantly higher and 18:0, 22:5n-3, and 22:6n-3 lower than in both the forestomach and the glandular part. The forestomach had, on the other hand, significantly lower level of 16:0 compared to both mucosa and the glandular part (Table 2).

DISCUSSION

The observations made in this study illustrate that the functionally most active part of the stomach, the gastric mucosa,

TABLE 2
Fatty Acid Composition of Phosphatidylethanolamine for All Stomach Parts of Animals Fed Standard Diet[a]

Fatty acid	Mucosa	Glandular part	Forestomach
16[b]	6.23 ± 0.41	12.59 ± 0.47a	13.57 ± 0.17a
16:0	7.75 ± 0.45	7.06 ± 0.13	4.57 ± 0.07a,b
16:1	—	0.95 ± 0.09	0.16 ± 0.16b
18[b]	8.69 ± 1.02	13.03 ± 0.78a	10.98 ± 0.20
18:0	8.07 ± 0.44	11.20 ± 0.50a	12.08 ± 0.35a
18:1n-9	20.02 ± 0.42	5.04 ± 0.08a	5.43 ± 0.07a
18:1n-7	1.88 ± 0.11	1.96 ± 0.07	1.51 ± 0.04a,b
18:2n-6	11.90 ± 0.67	5.32 ± 0.63a	5.07 ± 0.13a
20:4n-6	29.15 ± 0.92	29.19 ± 0.38	31.63 ± 0.53
20:5n-3	0.42 ± 0.19	—	—
22:5n-3	0.07 ± 0.07	1.67 ± 0.16a	2.17 ± 0.27a
22:6n-3	1.17 ± 0.08	2.98 ± 0.20a	3.76 ± 0.14a,b
Others[c]	4.65 ± 0.85	9.01 ± 0.79	9.07 ± 0.81

[a]Values are the means ± SE of five preparations. Three stomachs were pooled per preparation. a, $P < 0.05$ vs. mucosa; b, $P < 0.05$ vs. glandular part.
[b]Derivative of fatty aldehyde.
[c]Fatty acids which are below 0.3% of total fatty acids.

had the lowest level of 20:4n-6 in PC, followed by the corresponding stomach wall or glandular part, whereas the forestomach had the highest level of 20:4n-6. The level of 20:4n-6 was thus lowest where the turnover can be expected to be greatest and reflects the availability of 20:4n-6. The level of 20:4n-6 in PE was, on the other hand, identical in gastric mucosa and the stomach wall of both the lower and upper parts.

The availability of arachidonic acid in gastric phospholipids plays an important role in production of prostaglandins and other prostanoids. The ability of gastric mucosa to resist injury induced by luminal irritants is influenced by prostaglandins. Prostaglandins and nitric oxide influence the various components of mucosal defense: they inhibit acid secretion, stimulate mucus and bicarbonate secretion, elevate mucosal blood flow, and accelerate the healing of ulcers (10). Among the prostanoids are also lipid mediators of inflammation, such as leukotrienes and thromboxane, which may contribute to the pathogenesis of gastric ulcers. *Helicobacter pylori* has been identified as a causative agent in active chronic gastritis, but these bacteria also can produce inflammatory lipid mediators such as platelet-activating factor (11).

The major phospholipids of the stomach wall contained significantly higher levels of satuated fatty acids, 16:0 and 18:0 in PC and 18:0 in PE than in the mucosa. The levels of 18:1n-9 and 18:2n-6 in PC or PE were two to four times greater in mucosa than in the stomach wall. The significance of these differences in fatty acid composition is not known. Low levels of docosahexaenoic acid were observed in PC and somewhat higher levels in PE of both mucosa and the stomech wall. Eicosapentaenoic acid was practically absent in these phospholipids.

Conclusion: The availability of arachidonic acid was markedly different in various parts of the stomach. The level of arachidonic acid was significantly different in PC of the three main parts of the stomach: the mucosa, the underlying glandular part and the forestomach, with the lowest level observed in mucosa. The level of arachidonic acid in PE was, on the other hand, identical in mucosa and the stomach wall.

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INTRODUCTION

Fatty Acids and Lipids from Cell Biology to Human Disease

The supplement of this issue of *Lipids* on "Fatty Acids and Lipids from Cell Biology to Human Disease" consists of the proceedings of the 2nd Congress of the International Society for the Study of Fatty Acids and Lipids (ISSFAL). The congress took place on June 7–10, 1995, at the Natcher Conference Center at the National Institutes of Health in Bethesda, Maryland. The scientific program consisted of four plenary sessions, eight symposia, and two workshops. The program was structured to emphasize the basic mechanisms involved in the physiological, pharmacological, and pathological functions and effects of fatty acids and lipids, as well as their clinical effects on growth and development and chronic diseases. The composition of the food supply in terms of fatty acids and lipids, the importance of the appropriate ratio of n-6/n-3 fatty acids, *trans* fatty acids in human milk, and the effects of fat substitutes were also included.

The congress consisted of 77 invited papers and oral presentations selected from the submitted abstracts, and 185 posters. There were two workshops: one on "Maternal Fatty Acid Requirement During Pregnancy and Lactation," and the other on "Dietary Fatty Acids, Carnitine, Mitochondria, and Chronic Disease: The Critical Lifelong Importance of the Weaning Period." The keynote address given by Barton Childs on "The Logic of Disease" focused on individuality, and the first plenary session was on "The Role of Fatty Acids in Gene Expression." This area of research is expanding rapidly and may explain part of the mechanisms by which eicosapentaenoic acid and docosahexaenoic acid have beneficial effects in cardiovascular disease by a decrease in fatty acid synthase mRNA, and in inflammatory and autoimmune disorders (arthritis, ulcerative colitis) by decreasing IL-1β mRNA. The area of inborn errors of metabolism in fatty acid synthesis and degradation is just beginning to claim its proper place, which for so long has been dominated by genetic defects of amino acid metabolism. Major advances have taken place in our understanding of brain function due primarily to improved methods.

The information from the epidemiological studies and our increased understanding of the principal mechanisms involved indicate that it is now timely to begin intervention studies (double blind controlled clinical trials) on the role of fatty acids in the prevention and treatment of coronary heart disease, hypertension, and cancer. Intervention trials are also needed in inflammatory and autoimmune diseases and disorders, such as arthritis, ulcerative colitis, psoriasis, and others.

These proceedings were edited by Norman Salem, Artemis P. Simopoulos, Claudio Galli, and Michel Lagarde, who also served as cochairpersons of the congress, and by Howard Knapp, the editor-in-chief of *Lipids*. The papers from the four plenary sessions are followed by the papers presented at the eight symposia. The editors are grateful to the authors for their prompt submission of the papers, to the reviewers for their diligence in reviewing the manuscripts, and to Dr. Howard Knapp and the editorial office of *Lipids* for their assistance in publishing this special supplement to *Lipids* in a timely manner.

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A Logic of Disease

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I was very flattered to be asked to open these proceedings. I know too little about fatty acids and their role in homeostasis, and yet my job is to say something that unifies the program, and, if I'm lucky, something that may come to mind now and then as the meeting goes on. Ordinarily these remarks would be made by one of you, who would distill and present the essence of the field, but your program committee has decided upon an experiment. The title of the meeting is Fatty Acids and Lipids, from Cell Biology to Disease, and the experiment consists of a test of the idea that there are some general rules which, transcending fatty acids and lipids, guide us along the path from cell biology to aberration of any homeostatic system, and that allow us to think synthetically as well as analytically.

When biologists say they seek to learn how things work, they mean first, how structures fit together to form homeostatic devices that integrate to form independent, individual, open systems, and second, how nearly these open systems approximate to the goal of an adapted species. So each gene controlled unit step of homeostasis serves both an individual and an evolutionary purpose, and by virtue of "how they work" in both senses, they determine degrees of congruence of open systems with the environments within which they maintain themselves, reproduce and encounter the rigors of natural selection. That is, however reductionist the study of how things work, be they fatty acids or lipids; whatever is found must fit in with the ideas of natural selection and evolution.

Degrees of congruence implies variable adaptation to include also incongruence, or failure of one or more unit steps of homeostasis to meet demands imposed by the environment. So incongruence, some of which leads to disease, is continuous with congruence, and so its logic must also derive from natural selection and evolution.

Nothing could be more alien to the thinking of most physicians, including those educators who perceive the body as a machine that the doctor fixes when it breaks. No doubt this idea has stimulated some truly spectacular triumphs, attained because medicine has drawn so extensively upon biology to describe proximate causes and to illuminate pathogenesis, even to part a veil beyond which we seem to see a limitless progress wherein the concept of cure is prominently enshrined.

But curiously, in the face of so much success, medical educators are distressed. They wonder how students can surmount the mass of facts. They worry about the withering of the art of medicine in the face of an intervening technology, and they perceive a loss of coherence in the diversity of specialism. These are serious criticisms that imply that a medical student, overwhelmed by the volume of facts as soon forgotten as learned, will in a triumph of training over education, of information over knowledge, succumb to technology for diagnosis and to prescribed routines for treatment.

Efforts to reverse these trends seem to be directed to details rather than to an examination of the metaphor of the machine. But some have begun to recognize a conceptual alternative based on a universal language that embraces all of biology and that has the power of coherence to inform all branches of medicine. It is the language of the DNA, wherein individual ontogenies are expressed in phylogenetic terms. To be reconciled to the environment, each open system draws upon the wisdom of its own genome and its own development, both already adumbrated in the phylogenetic past. This alternative provides a synthetic outlook, offering answers to such questions as why disease exists, specifically who is at risk, and for what and when in a lifetime. And it is in answers to these questions, upon which the machine mentality is mute, that we perceive a basis for attaining a higher goal than that of cure; that of prevention.

How does this language inform a logic of disease that faces squarely the educators' concerns? And how does such a logic comprehend the inborn errors and the complex diseases that are the subject of some of our program?

We might begin by observing that every human being leads two lives. In one we are creators, repositories and transmitters of the genetic variability required to maintain the species, while in the second we use our endowment and our experiences to evolve, through development, an individuality whereby we make our place in the world. In the first we serve the species, in the second ourselves, and in both are found incongruencies that lead to disease. That is because, in serving the species, variability of both genes and culture is engendered in indifference to the welfare of particular individuals. Life at meiosis, wherein are created mutants varying from gains or losses of whole chromosomes to noncoding substitutions, is completely uncoupled from life after fertilization. And while the rules of cultures evolve in consonance with the needs of the societies they serve, they cannot suit every individual. So the incongruencies that lead to disease originate in the need for adaptive variability in both genes and culture.

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This idea of incompatibility between phylogeny and cultural evolution is no part of the general lore of medicine and is wanting in the metaphor of the machine, which, in concentrating on pathogenesis and cure, allows us to forget our role as representative of the species to concentrate on that of the cultural self. But that Nature doesn't overlook human nature is demonstrated in predictions about disease that flow from its evolutionary origins, and that furnish the basis for its logic. None of these predictions is anticipated by the machine mentality.

What is predicted? First, since genes control the unit steps of homeostasis and disease results from disadaptive variations of one or more such unit steps, then all disease should be perceived as a consequence of genetic vulnerability, whether of the whole species or of individuals. Such vulnerabilities are popularly known as inborn errors, and their number is constrained only by the number of genes, and the number of disorders they characterize is a property of the manifold ways their effects can be integrated to produce signs and symptoms.

Second, given the extravagance of mutational variety generated at meiosis, there should be a gradient of selective effect maximal at conception and diminishing throughout life, and conversely, we should expect for experiences of the environment a similar gradient, measured in kind, intensity and duration, that is least at conception and increases throughout life; and these gradients should be reflected in the qualities of the diseases that characterize ontogeny and should be demonstrable in the context of the lives of a cohort of conceptuses followed from the beginning until the death of its last member.

Third, the gradient of selective effect together with the qualities of ontogeny predict that when diseases are considered in relation to each other in regard to the complexity of their origins, the time of their onsets and their threat to life and reproduction, there should emerge a pattern of continuity of these properties easily demonstrated in the fates of the cohort of conceptuses.

Fourth, the exuberance of mutational events at meiosis assures numerous mutations for every locus so that genetic heterogeneity should be assumed until shown not to exist. Heterogeneity of provocative experiences is not less to be anticipated.

Fifth, there should be an inverse relationship between selective intensity and gene frequency—and so disease. Genes high in the gradient, and their diseases—usually called "genetic," should be rare, those low in the gradient common and unassociated with disease. In between are genes that are conditional with regard to disease, contributory in some individuals, innocuous in others and often depending upon experiences of the environment.

Sixth, when differences in frequency and expression of disease by sex is not explained by sex-linkage, it may be that one sex is protected by some difference in homeostasis, in which case a greater concentration of risk factors, both genetic and experiential, is expected in affected individuals of the protected sex.

Seventh, the relative contribution to disease of genes and experiences varies reciprocally in populations, depending upon the distributions of both genes and provocative experiences. And, in conformity with the gradient of selective effect, the heritability of disease declines with age at onset.

Eighth, all of these predictions should be fulfilled within diseases no less than between.

And ninth, exceptions exist to all of these rules. Such a remark is saved from banality by observing that each human being, in being unique, is exceptional in some way. And in any case, nature the tinkerer preserves what works regardless of generalizations.

These are all in the nature of hypotheses. What evidence can be adduced to test them? First, the idea of the unit step of homeostasis, or, more familiarly, the inborn error, as a central theme in medicine, is supported in two ways: (i) the classical inborn errors continue to be described at an exponential rate (Fig. 1), and (ii) the idea that any gene is susceptible to mutation is attested by the demonstration of monogenic disease due to abnormality of so many different kinds of gene product, including growth factors, antibodies and cytokines, enzymes, receptors, signal transducers and transcription factors (1,2). In addition, numerous mutants in different constellations are shown to be associated with multifactorial disorders, genes not of overriding authority but contributing each in its unique way to the disruption of homeostasis and leading to diabetes, hypertension, coronary artery disease and so on. Acceptance of these within the rubric of the inborn error is demonstrated in the inclusion in the book *The Metabolic and Molecular Basis of Inherited Disease*, of cancer, congenital anomalies, diabetes, and gout (1). The editors, whose book is already 4600 pages, are aware that in proposing that all disease has a genetic, molecular, and metabolic basis, they have got a tiger by the tail.

Next, the gradient of selective effects is demonstrated in a U-shaped distribution of mortality rates according to age. The causes of death on the two sides of the antimode are different; prepubertal life on the left, adult life and aging on the

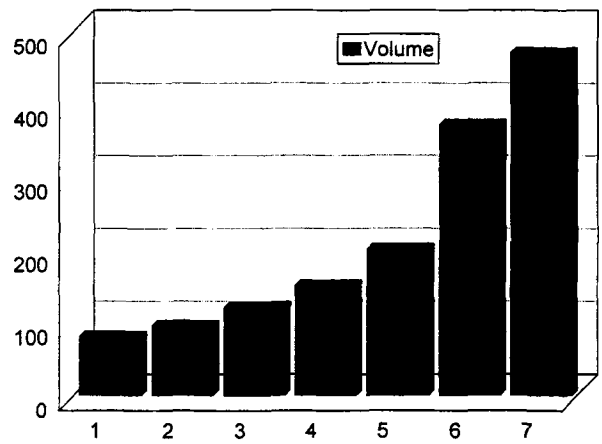


FIG. 1. Classical inborn errors are being identified at exponential rates.

right. Table 1 gives further evidence bearing on these hypotheses. In support of the gradient is the observation that the most burdensome diseases occur early in life. They are the monogenic inborn errors, disorders of high diagnostic specificity, many affected relatives, uninfluenced by social conditions, very resistant to treatment and, significantly, often preventable only by choosing not to have babies or by eliminating the affected fetus. The other column shows the reciprocal position of diseases of adult and late life; multifactorial, genetically more heterogeneous, long latency, lower diagnostic specificity, lesser burden, more responsive to social and temporal change and far more responsive to treatment and, especially, to prevention.

This comparison also brings out the overlap and continuity of disease (Fig. 2). In this hypothetical diagram, the left arm represents the mortality of intrauterine disease; a time of life of little interest to the machine mentality, but at the very core of our understanding of disease. Something like 75% of conceptuses are presumed to have been lost *in utero*. Before birth, most of our cohort is gone, much of it before implantation. That which can be recognized is due to chromosome abnormality (3), but given what we know about meiosis, there should be all sorts of defects of unit steps of homeostasis, engaged perhaps in DNA replication, in the cell cycle, cell division or the control of growth; for example an irrepressible oncogene might do a lot of damage.

So this phase of life is marked by selective purification, but since nature never reaches for perfection, there is overlap, a backwash of abnormality, mostly anomalies, that reach term. Some of these die quickly, but some do not declare themselves until later, if then. For example, an absent kidney might be noticed only in the face of infection, or trauma or not at all.

A second phase is initiated at or before birth when the fetus' efforts to assume metabolic self-control bring out all sorts of inborn errors, and while 95% of these are apparent by puberty, only 1% has onset after age 40 (4). So, while monogenic diseases characterize prepubescent life and multifactorial disorders distinguish later life, the slide shows overlap; monogenic disorders in later life and multifactorial in early life; evidence for both the gradient of selective effect and for the continuity of disease. In addition, the gradient is not exactly monotonic; there is an effect of ontogeny (4). That is, the bulk of the genetic effect is always exerted at the beginning of each developmental phase, that of the environmental effect at the end. For example, mortality at the end of intrauterine life is due to multifactorial anomalies, birth injury, infections, placental imperfection and the like, as opposed to the largely monogenic and chromosomal effects at the start. Similarly monogenic disorders distinguish early post-natal life, accidents and multifactorial disorders distinguish childhood and adolescence, and, while there is some resurgence of genetic effect in adult years, in old age the main contributor to disease is aging itself.

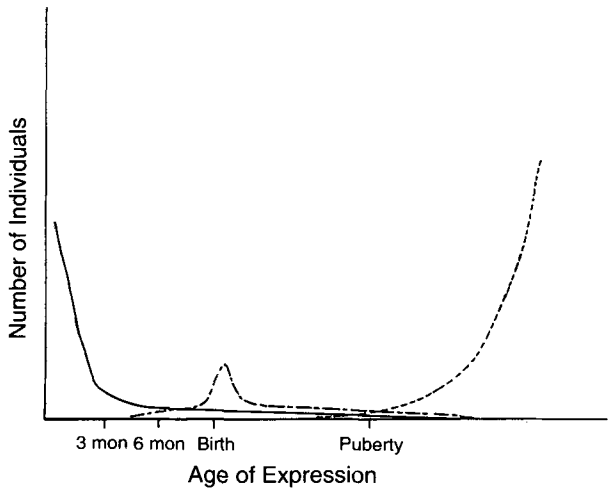


FIG. 2. Causes of mortality characteristic of three different phases of development.

TABLE 1
Differences Between Prepubertal and Postpubertal Diseases

	Prepubertal	Postpubertal
Mode of inheritance	Monogenic	Multifactorial
Age of ofset	Early	Late
Frequency	Rare	Frequent
Latency	Short	Long
Affected relatives	Numerous	Few
Diagnostic specificity	High	Low
Number of diseases	Very many	Fewer
Burden	Great	Less
Sex differences	Occasional	Frequent
Influence of migration	No	Yes
Secular change	No	Yes
Effects of SES	Some	More
Success in treatment	Some	More

The sixth hypothesis suggested that representatives of the sex with the lower incidence should require more provocation to express the disease. This prediction is demonstrated in women with premature heart attacks who, in addition to a higher mortality and strong family history, nearly always have all of the other risk factors, too (5). But once past the menopause, the risk for women is the same as that for men; there has been a decline in heritability (6).

The seventh hypothesis, that the heritability of disease decreases with age, is exemplified in greater secular effects. Prominent among these are the experiences of the Pima Indians of the United States and the Polynesians of Nauru where affluence and Western ways brought into full bloom underlying genetic propensities to type II diabetes and gout among other conditions (7). No doubt there were a few cases of diabetes and gout before Western habits, and no doubt they were strongly familial, but the Western habits played over lesser genetic vulnerabilities and the incidence of the diseases rose. These are cautionary tales. One wonders whether survivors represented in the recent decline in heart attacks among men were those with relatively less genetic variability. The rule is

that preventive measures work best in the least vulnerable, while the most vulnerable are the most resistant.

Figure 3 demonstrates the eighth hypothesis, that all characteristics are present within diseases as well as between. Gout is a disorder with onset from infancy to old age. Degree of familiarity, severity and serum urate levels all decline with age at onset and, when causes are scrutinized, we see among the very young several monogenic, severe, nearly untreatable inborn errors in which urate is over produced (8). In the middle years, classical gout, due to failure in excretion of urate, is strongly familial but generally treatable, and among the elderly are patients taking diuretics for hypertension, still familial but requiring the medication to bring on the symptoms and easily treated by the simple expedient of stopping the diuretic. Better yet, a family history of gout might be a reason not to use the diuretic in the first place. The prevalence of heterogeneity is also illustrated. Gout is a word, not a disease, and a word that loses specificity with age.

Infections and cancer are not excepted in the logic (9). Infections are a result of the actions of two genotypes, each capable of defense against the other. The human defense is massive and, in comparison, static, and vulnerability is represented in numerous inborn errors of the multi-tiered immune system, as well as in perfectly normal elements of homeostasis that become points of weakness to microorganisms whose mutational versatility defines them as such. The position of microorganisms is the reciprocal, a genetic competence to adapt rapidly to the prevailing human defense and to antibiotics, and a practical response to selection in which their "inborn errors," unlike ours, are swiftly disposed of. And cancer is associated with mutants—somatic mostly, but not always—in multiple unit steps of the homeostasis of growth (10). Both fulfill the hypotheses of the genetic gradient, continuity, heterogeneity and so on.

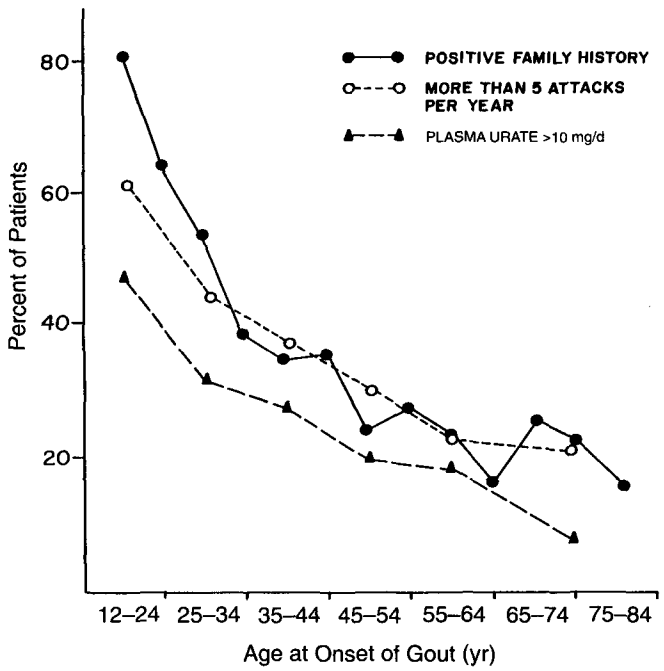


FIG. 3. The severity of gout and its rate of recurrence in families decline with age at onset.

Finally, how is this alternative superior to the metaphor of the machine, the latter having advanced our cause so efficiently? I suggest it is not superior, but inclusive; and in its emphasis on individuality and change during life, it directs our attention to prevention. If we see the body as a machine, we begin with the assumption that one way of fixing the break will do for all, although we move to more individual application when required. In contrast, the alternative begins with the assumption of individuality in both treatment and prevention; one prevention for all is likely to represent privation for the nonvulnerable and to be insufficient for the susceptible. We are told that the genome initiative will reveal everything about our genetic propensities, but until we modify our thinking to include the individuality of both genotype and ontogeny, we shall lack the insight to use the information safely and wisely.

So now the experiment is finished and the time has come when it must bear the pitiless scrutiny of judgment and interpretation. If successful, there may be some reverberations, perhaps distant, in the thinking that will characterize the meeting.

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Polyunsaturated Fatty Acid Regulation of Hepatic Gene Transcription

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ABSTRACT: Polyunsaturated fatty acids (PUFA) of the n-6 and n-3 families inhibit transcription of a number of hepatic lipogenic and glycolytic genes, e.g. fatty acid synthase. In contrast, saturated and monounsaturated fatty acids exert no suppressive action on lipogenic gene expression. The unique PUFA regulation of gene expression extends beyond the liver to include genes such as adipocyte glucose transporter-4, lymphocyte stearoyl-CoA desaturase 2, and interleukins. Some of the transcriptional effects of PUFA appear to be mediated by eicosanoids, but PUFA suppression of lipogenic and glycolytic genes is independent of eicosanoid synthesis and appears to involve a nuclear mechanism directly modified by PUFA. With the recent cloning of a fatty acid–activated nuclear factor termed peroxisome-proliferator–activated receptor (PPAR) has come the suggestion that PPAR may be the PUFA response factor. This review, however, presents several lines of evidence that indicate that the PPAR and n-6 and n-3 PUFA regulation of lipogenic and glycolytic gene transcription involve separate and independent mechanisms. Thus PPAR appears not to be the PUFA response factor.
Lipids 31, S-7–S-11 (1996).

Several different disease states, including atherosclerosis, noninsulin-dependent diabetes (NIDDM), and certain types of cancer, have been linked to type and amount of dietary fat (1–4). Dietary fats appear to influence the onset and progression of these various diseases by exerting an effect at two levels: (i) altering fatty acid composition of membrane phospholipids (1,5,6) and (ii) regulating nuclear events that govern gene transcription (7–12). Thus the beneficial (e.g. reduced blood triglycerides) as well as detrimental (e.g. impaired glucose tolerance) effects that dietary fats exert on various diseases may involve a combination of interactive regulatory mechanisms, i.e. rapid and direct fatty acid regulation of gene transcription accompanied by long-term adaptive modulation of membrane composition leading to changes in hormone signaling. The role of membrane phospholipid fatty acid composition in hormone signaling and receptor activity has been extensively reviewed (5,6), but the notion that fatty acids rapidly and directly alter transcription of specific genes is a recent discovery. For this reason, this review focuses on the effects that dietary n-6 and n-3 fatty acids exert on gene expression.

Polyunsaturated fatty acid (PUFA) regulation of gene transcription. The unique regulatory nature of dietary polyunsaturated fats was first observed by Allmann and Gibson (13) who discovered that within 2 d of adding 2% 18:2n-6 to a high-carbohydrate, fat-free diet for mice, the rate of hepatic fatty acid biosynthesis and the activities of fatty acid synthase (FAS), malic enzyme, and glucose-6-phosphate dehydrogenase were depressed by 70%. In contrast, supplementing the carbohydrate-rich diet with palmitate or oleate had no effect on hepatic lipogenesis or activities of lipogenic enzymes. Over the past 25 years, several investigators demonstrated that dietary polyenic fatty acids of the n-6 and n-3 fatty acids suppress hepatic lipogenesis, whereas saturated and monounsaturated fatty acids have no inhibitory capabilities (7,8,10,11,14–29). This action of n-6 and n-3 fatty acids is independent of carbohydrate intake (16,25,27,28) and occurs at a level of fatty acid intake that is four- to five-fold greater than that needed to fulfill the essential fatty acid requirement for optimal growth (28).

This well-documented suppression of hepatic *de novo* fatty acid biosynthesis by dietary n-6 and n-3 PUFA reflects the unique ability of these fatty acids to reduce hepatic activities of enzymes in the fatty acid biosynthetic pathway (30). Such suppression of enzymatic activities does not represent a fatty acid–mediated impairment in enzyme catalytic efficiency (e.g., fatty acyl-CoA inhibition of acetyl-CoA carboxylase), but rather reflects a decrease in hepatic enzyme content caused by a PUFA-mediated suppression of enzyme synthesis (28). A clearer understanding of how n-6 and n-3 PUFA decrease

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Abbreviations: ETYA, eicosatetraynoic acid; FAS, fatty acid synthase; NIDDM, noninsulin-dependent diabetes mellitus; PPAR, peroxisome-proliferator–activated receptor; PUFA, polyunsaturated fatty acids.

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enzyme synthesis came in 1990 when Clarke and associates (10) demonstrated that PUFA decreased hepatic FAS mRNA levels by inhibiting the rate of gene transcription (7,8,11,12).

The transcriptional response to dietary PUFA is very rapid (10,29). For example, supplementing a high-carbohydrate, fat-free diet with 5% (w/w) menhaden oil results in a 60% suppression of transcription for the FAS, pyruvate kinase, and S_{14} genes in <3 h (29). Moreover, removal of PUFA from the diet leads to a rapid (<3 h) induction of gene transcription (10). Such a rapid response strongly suggests that PUFA directly modulate gene transcription rather than exert their influence by modifying membrane lipid fatty acid composition and altering hormone release or signaling. In support of this conclusion, we have found that hepatic insulin signaling mechanisms are not different in rats fed polyunsaturated fat (i.e., safflower oil) or saturated fat (i.e., tallow) (28). Moreover, selective control of lipogenic gene expression can be recreated with hepatocytes in monolayer culture, which eliminates from consideration modifications of hormone release (7,29).

Since the discovery that n-6 and n-3 PUFA suppress transcription of the hepatic FAS gene, several additional hepatic and nonhepatic genes were found to be transcriptionally regulated by PUFA. For example, dietary n-6 and n-3 PUFA suppress transcription of several lipogenic genes, including acetyl-CoA carboxylase (21,22), glucose-6-phosphate dehydrogenase, malic enzyme (23,25), and stearoyl-CoA desaturase 1 (33), also 20:4n-6 and 20:5n-3 are potent inhibitors of glycolytic gene expression (29). While dietary fat suppression of lipogenic and glycolytic genes is a specific response to n-6 and n-3 PUFA, both monounsaturated and PUFA appear to be able to induce genes coding for peroxisomal genes, notably acyl-CoA oxidase (34). Induction of the acyl-CoA oxidase gene, however, is greater with dietary fish oils than with dietary corn oil (Clarke, S.D., and Jump, D.B., unpublished data). It remains to be determined if this greater responsiveness to fish oil is due to the high amount of 20/22-carbon PUFA, or if it is something specific for n-3 polyenoic fatty acids. Induction of peroxisomal genes and the accompanying peroxisomal proliferation may explain the thermogenic response that results from feeding rats high-PUFA diets.

Recently, PUFA inhibition of gene transcription was found to extend to adipocytes (35) and mouse lymphoma cells (36). In preadipocytes, n-6 PUFA accelerated fat cell differentiation and induced genes of terminal fat cell differentiation such as the adipocyte fatty acid binding protein (37–40). In mature fat cells, however, 20:4n-6 suppressed expression of insulin-sensitive glucose transporter-4 while increasing expression of glucose transporter-1 (35). This effect of 20:4n-6 on adipocyte glucose transport extends to 20:5n-3 (Pekala, P.H., personal communication). Thus a logical extension of these observations is that diets high in 20:4n-6 or 20:5n-3 may lead to an impairment of insulin-dependent glucose uptake in adipose tissue and thereby exacerbate NIDDM. Clearly, PUFA exert a very unique influence of gene transcription in a number of different cell types, which may explain the numerous beneficial as well as detrimental effects attributed to various dietary fats.

Structural characteristics of a PUFA inhibitor. Unfortunately, the specific fatty acid mediator responsible for regulating gene transcription is unknown. Analyses of double-bond positioning and configuration, however, have yielded some insight into the nature of the PUFA inhibitor. First and foremost, an inhibitor fatty acid must contain two double bonds, and these must be positioned between carbons of 9–10 and 12–13 of an 18-carbon fatty acid (28). Dieneoic fatty acids (i.e., 5,13–22:2) without this positioning are ineffective as transcriptional inhibitors (28). In addition, at least one of these double bonds must be *cis*, but the other can be in the *trans* configuration (28). Such structural requirements for an inhibitory fatty acid suggested that the PUFA inhibitor may be an eicosanoid. Numerous studies with a wide array of eicosanoid inhibitors, however, have failed to prevent PUFA suppression of lipogenic gene expression (18,31,32). Interestingly, supplementing the arachidonic acid analogue, eicosatetraynoic acid (ETYA), to a diet containing corn oil or 18:2n-6 prevented PUFA suppression of lipogenic genes (15,25,32), but feeding 20:4n-6 with ETYA reinitiated PUFA suppression of fatty acid biosynthesis (32). An examination of hepatic fatty acid composition indicated that ETYA suppressed Δ6 desaturase activity. Thus, in addition to requiring double bonds at carbons 9 and 12, it appears that a double bond must also be introduced at position 6 of an 18-carbon PUFA (15,25,32). It remains unclear, if Δ5 desaturase activity is essential for the PUFA effect.

Inhibitory potency of n-9, n-6, and n-3 fatty acids. Saturated and monounsaturated fatty acids have little or no capacity to suppress expression of hepatic lipogenic and glycolytic genes (28). In fact, under some conditions, monounsaturated and saturated fatty acids have been found to enhance expression of genes encoding lipogenic enzymes (7,16). This stimulatory response may be due to a competitive action of the noninhibiting fatty acid with the suppressive PUFA metabolite.

Opinions vary with respect to the relative antilipogenic potency of n-6 and n-3 fatty acids. In general, 18:2n-6 and 18:3n-3 are equipment suppressors of FAS, glucose-6-phosphate dehydrogenase, and malic enzyme gene expression (16). Menhaden oil, however, suppresses hepatic FAS expression two to three times more effectively than does safflower oil (28). The greater potency of fish oil is observed as a two-fold greater maximal suppression of FAS activity and as a threefold lower requirement for fish oil to achieve 50% maximum inhibition (28). In addition, fish oil is a far more potent inducer of hepatic peroxisomal acyl-CoA oxidase expression than is corn oil (Clarke, S.D., Turini, M., and Jump, D.B., unpublished data). Moreover, fish oil induction of acyl-CoA oxidase extends to several nonhepatic tissues, while corn oil appears to have little impact on acyl-CoA oxidase expression in nonhepatic tissues (Turini, M., and Clarke, S.D., unpublished data). A further difference between fish oil and vegetable oil lipids is that fish oil markedly suppresses expression of hepatic pyruvate kinase, while vegetable oils rich in 18:2n-6 fatty acids appear to only minimally impair glycolytic gene expression (10, 12). These differences in gene responsivity may suggest

that transcriptional regulatory mechanisms vary among various affected genes. To partially address this question, we compared the relative potency of 20:4n-6 with comparable levels of 20:5/22:6n-3 supplied by fish oil (28). Interestingly, fatty acid synthase expression was equally reduced by 20:4n-6 or fish oil (28). Thus, the greater inhibitory effectiveness of fish oil over vegetable oils is in part due to the presence of 20/22-carbon PUFA in the fish oil. It remains to be determined, however, if pyruvate kinase and acyl-CoA oxidase are equally responsive to dietary 20:4n-6 and 20:5n-3.

The differential response of pyruvate kinase and FAS to 18:2n-6, and presumably 18:3n-3, vs. the fatty acid products of Δ6 desaturase may have significance for dietary recommendations. Dietary oils rich in 18:2n-6 may suppress hepatic lipogenesis and lower hepatic triglyceride production, but because 18:2n-6 is less effective as a suppressor of glycolytic enzyme expression, hepatic glucose metabolism may continue and lead to an increased substrate availability for ketone synthesis. Such a feature is clearly undesirable in an NIDDM subject. Conversely, fatty acid products of Δ6 desaturase appear to coordinately suppress glycolytic and lipogenic genes. In this way, hepatic glucose oxidation may be reduced, but the glucose may be repartitioned into hepatic glycogen synthesis (3). Thus both 20:4n-6 and 20:5n-3 may potentially function equally well as fuel-partitioning agents in modulating hepatic glucose metabolism and improving the NIDDM condition.

PUFA-response region and possible PUFA-responsive transcription factors. While the identity of the fatty acid effector has not been established, significant progress has been made in localizing potential *cis*-acting elements affected by dietary PUFA. Using transfection analysis, Jump and associates (11,12,41) demonstrated that polyenoic n-6 and n-3 fatty acids suppressed chloramphenicol acetyltransferase expression in cultured rat hepatocytes that were transfected with vectors containing –4315 to +19bp and –4300 to +12 b.p. of the S_{14} and pyruvate kinase genes, respectively. Additional 5′-deletion studies revealed that the PUFA-response region mapped to sequences located at –220 to –80 b.p. and –197 to –96 b.p. of the S_{14} and pyruvate kinase genes, respectively (11,12). Using linker scanner mutation analysis, the precise PUFA- response sequence was determined to be located in the LS-4 (–143 to –135 b.p.) and LS-5 (–130 to –123 b.p.) regions of the pyruvate kinase promoter (12). Interestingly, these regions are part of the site thought to mediate the insulin/carbohydrate induction of transcription for the pyruvate kinase and S_{14} genes (11,42). In light of this observation, it is interesting to speculate that PUFA may mediate its suppressive effects on lipogenic and glycolytic gene expression by interfering with the nuclear action of the insulin-signaling cascade. Such a PUFA/insulin interaction, however, may not be universal because the FAS gene also contains an insulin/carbohydrate response region in its proximal promoter (43), but PUFA do not regulate FAS gene transcription at this site (Clarke, S.D., Turini, M., Jump, D.B., and Thelen, A., unpublished data).

Because of the rapidity with which dietary PUFA suppress transcription of lipogenic and glycolytic genes, we proposed that PUFA may be a ligand for a specific nuclear transcription factor that functioned as a dominant-negative suppressor of gene transcription (28,30). Our concept of a PUFA-binding protein was strengthened by the discovery and cloning of a family of nuclear transcription factors termed peroxisome-proliferator–activated receptors (PPAR) (39,44–50). PPAR are members of the steroid-like receptor superfamily and are located in the nucleus of a number of tissues, including the liver and adipocytes (44–50). Activators of PPAR include a diverse group of compounds such as amphipathic carboxylates, herbicides, plasticizers, and most importantly fatty acids (39,44–50). Interestingly, peroxisomal-proliferating agents, such as clofibrate, reportedly suppress expression of FAS and inhibit hepatic lipogenesis (34). Moreover, the time course for induction of the peroxisomal enzyme acyl-CoA oxidase by clofibrate is comparable to that for suppression of FAS expression (34). Collectively, these data led many investigators to suggest that PPAR is the *trans*-factor responsible for mediating the fatty acid regulation of gene transcription (39,44–50). A number of recent *in vivo* and *in vitro* studies, however, fail to support the PPAR hypothesis.

First, the PUFA-response region in the proximal promoter of the pyruvate kinase and S_{14} genes binds several transcription factors, but the region does not contain a PPAR-response element nor does it bind PPAR (12,41). In fact, linker scanning mutation analysis of the PUFA-response region in the pyruvate kinase gene indicated that PUFA affected the HNF-4 site of the PUFA-response region (12). It is noteworthy that HNF-4, like PPAR, is a member of the steroid-receptor superfamily, but there is no evidence at this time indicating that fatty acids regulate HNF-4 or alter interactions with its response element. Moreover, the putative HNF-4 target site for PUFA regulation of gene transcription may not be common to all glycolytic and lipogenic genes. As an example, the FAS gene contains a carbohydrate-like response sequence in its proximal promoter and in the first intron, but functional analysis studies with fatty acid synthase gene sequences ranging from –2195 to +750 b.p. have failed to detect a PUFA-response region even though PUFA are potent inhibitors of fatty acid synthase gene transcription (Clarke, S.D., Turini, M., Jump, D.B., and Thelen, A., unpublished data). Thus it is likely that PUFA regulate gene transcription in multiple ways and/or at multiple sites in genes encoding lipogenic and glycolytic enzymes.

Secondly, the strongest evidence suggesting tha fatty acids regulate gene transcription by modulating PPAR actions is derived from transcription *trans*-activation assays (45,48). In these assays, however, saturated, monounsaturated, and polyunsaturated fatty acids all have equal potency as activators of PPAR, and all induce PPAR-mediated gene transcription (45,48). Thus PPAR-like proteins do not appear to exhibit the selectivity required to explain PUFA control of gene expression. In addition, feeding mice the potent PPAR-activator ETYA induced peroxisome proliferation, as indicated by the significant rise in hepatic acyl-CoA oxidase mRNA level, but ETYA had no effect on FAS expression (Turini, M., and Clarke, S.D., unpublished data). Similarly, linoleic and

arachidonic acids, which potently suppress FAS gene expression, had no effect on expression of acyl-CoA oxidase (Turini, M., and Clarke, S.D., unpublished data). Thus induction of peroxisomal and suppression of lipogenic and glycolytic genes appear to involve independent mechanisms.

We have known for nearly 30 yr that dietary polyenic n-6 and n-3 fatty acids potently inhibit hepatic fatty acid biosynthesis. The teleological explanation for this unique action of PUFA resides in their ability to suppress the synthesis of n-9 fatty acids. By inhibiting fatty acid biosynthesis, dietary PUFA reduce availability of substrates for stearoyl-CoA desaturase (30) and in turn reduce availability of n-9 fatty acids for incorporation into plasma membranes. In this way, essential biological processes dependent on essential fatty acids (e.g. reproduction and transdermal water loss) continue to operate normally. Therefore, if essential fatty acid intake did not regulate n-9 FAS, survival of the organism would be threatened. During the past 20 yr, we have gradually elucidated cellular and molecular mechanisms by which dietary PUFA modulate fatty acid biosynthesis and n-9 fatty acid availability. Central to this mechanism has been our ability to determine that dietary n-6 and n-3 PUFA regulate transcription of genes coding for lipogenic and glycolytic enzymes (8,11,12). Moreover, we have found that dietary fish oils are more potent inhibitors of gene expression than are dietary vegetable oils, but this is due to the fact that fish oil contains a high amount of 20- and 22-carbon polyenoic fatty acid rather than due to intrinsic potency differences between n-6 and n-3 fatty acids.

The potential mechanisms by which n-6 and n-3 PUFA govern gene transcription are numerous, and it is unlikely that any one mechanism can fully elucidate the nuclear actions of PUFA. The difficulty in providing a unifying hypothesis at this time stems from (i) the many metabolic routes taken by PUFA on entering the hepatocyte and (ii) the lack of identity of a specific PUFA-regulated *trans*-acting factor. Nevertheless, a collection of information is available which has led us to propose the mechanistic scheme outlined in Figure 1. Clearly, PUFA are not only utilized as fuel and structural components of cells, but also serve as important mediators of gene expression (30). As regulators of gene expression, PUFA (or their metabolites) are thought to affect the activity of transcription factors, which in turn target key *cis*-linked elements associated with specific genes. Whether this targeting involves DNA–protein interaction or the protein/protein interactions of PUFA-regulated factors is unclear. A better understanding of the nuclear actions of PUFA will clarify the role of these compounds in lipid metabolism and lead to a better understanding of the role of PUFA in disease processes such as NIDDM and certain forms of cancer.

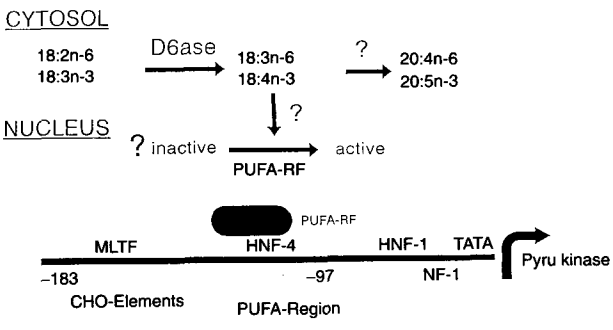


FIG. 1. Using the pyruvate kinase gene as a model, the potential mechanism by which polyunsaturated fatty acids (PUFA) may regulate hepatic gene transcription is outlined. The essence of the model is that Δ6 desaturation is essential for PUFA control of transcription and that desaturase products activate a hypothetical nuclear response factor that interacts with the HNF-4 *trans* factor to modulate transcription: D6ase = Δ6 desaturase; PUFA-RF = a hypothetical nuclear PUFA-response factor; MLTF, major late transcription factor.

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Effect of Polyunsaturated Fatty Acids in Obese Mice

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ABSTRACT: Genetically obese (ob/ob) mice display a variety of metabolic differences from lean litter mates. In the obese state, fatty acid desaturation-elongation in brown adipose tissue mitochondria is apparently altered, resulting in differences in membrane fatty acid composition. This change in membrane lipid environment appears to influence GDP binding and therefore the activity of the proton conductance pathway associated with regulation of energy expenditure in these animals. In liver, binding of insulin to the nuclear membrane is increased by feeding a high polyunsaturated/saturated (P/S) diet fat. Consumption of a high P/S diet decreased mRNA levels for fatty acid synthase, acetyl-CoA carboxylase, malic enzyme, and pyruvate kinase in obese and lean animals. Expression of mRNA for these lipogenic enzymes was higher in obese animals and suggests that obese mice may be resistant to polyunsaturated fatty acid feedback control of gene expression.
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Essential fatty acids (EFA) and nonessential fatty acids that can be synthesized *de novo* from acetyl-CoA are essential for energy balance, membrane biosynthesis, and other specialized functions. The dietary balance among these components affects partitioning of how each may be utilized on a whole-body basis (1). Intake of fatty acid affects molecular mechanisms involved in nutritional regulation of fatty acid synthesis (2), coordinate control of glycolytic and lipogenic gene expression (3,4), and transcription of stearoyl-CoA desaturase-2 gene (5).

Obesity is accompanied by abnormalities in systemic carbohydrate and lipid metabolism and in insulin secretion and insulin action. Phenotypic emergence of this genetic defect begins early in life and may profoundly alter development of adipose tissue toward excessive fat storage (6–8). The problem of obesity is essentially a problem of energy balance. Excess caloric intake relative to caloric expenditure leads to the storage of extra fat at various rates, depending on the degree of imbalance (9).

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Abbreviations: BAT, brown adipose tissue; EFA, essential fatty acids; FABP, fatty acid binding protein; GDP, guanosine 5′-diphosphate; NaTES, sodium N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid; ob/ob, genetically obese; PC, phosphatidylcholine; PE, phosphatidylethanolamine; P/S, polyunsaturated/saturated.

In the genetically obese (ob/ob) mouse, brown adipose tissue (BAT) appears to act primarily as an energy buffer in the overall regulation of energy balance (10). High metabolic efficiency associated with defective BAT thermogenesis (11) results in a positive energy balance, which may manifest itself in obesity. Although there are notable exceptions (12–14), in general these mice exhibit reduced responses to norepinephrine stimulation (15,16), even in the cold (17), with the consequence that ob/ob mice maintain a lower body temperature when mildly cold-exposed (16). This defect in cold-induced nonshivering thermogenesis appears to be coupled with a reduced capacity for diet-induced thermogenesis (18–20), a phenomenon characterized by elevated thermogenesis and higher metabolic efficiency. Dietary treatment can also augment BAT thermogenesis (21–24). A high fat diet rich in EFA has been shown to partially reverse the impaired response of BAT to norepinephrine (25). Evidence showing that a high-fat cafeteria diet can normalize low sympathetic nervous activity in BAT (26) suggests (i) that the ob/ob mouse may respond more rapidly to dietary fat than to dietary carbohydrate and (ii) that enhancement of BAT thermogenesis and increased metabolic efficiency in high-fat cafeteria-fed mice is integrally linked to the hypothalamus and the mechanism by which the hypothalamus senses energy intake (10).

Using diets of defined composition, it has been shown that BAT thermogenic activity, as determined by mitochondrial guanosine 5′-diphosphate (GDP) binding, can be influenced by fatty acid composition of diets consumed (22). This implies that the amount and type of dietary fatty acids are important in the regulating of BAT thermogenesis. Moreover, it suggests that a defect in BAT thermogenesis in the ob/ob mouse may also have its origins at the level of the inner mitochondrial membrane, the site of the 32,000 kD uncoupling protein that regulates energy dissipation across the membrane. Dietary fatty acids can alter mitochondrial membrane composition (27), and thus the lipid environment of the proton conductance pathway, or its purine nucleotide binding sites. This may be of significance in that other tissues in obese mice [i.e., liver (28,29) and epididymal fat pads (29)] show marked changes in the relative proportions of linoleic acid (18:2n-6) and arachidonic acid (20:4n-6) as obesity is expressed. This suggests that the obese genotype affects membrane fatty acid composition through alterations in the desaturation-elongation of n-6 fatty acids, presumably *via* effects on Δ6- and/or Δ5-desaturase activity.

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In preobese pups, increased lipogenic capacity of adipose tissue is due to preferential channeling of nutrients into fatty acids. A number of enzyme activities related to lipid storage are coordinately increased in a tissue-specific manner (30–32), predominantly in the liver. *De novo* fatty acid biosynthesis is catalysed by two enzyme systems that function sequentially: acetyl-CoA carboxylase and fatty acid synthase. At the primary site for *de novo* synthesis of long-chain fatty acids, most of the NADPH required is furnished via malic enzyme. Activities of acetyl-CoA carboxylase, fatty acid synthase, and malic enzyme are altered in the obese state (33–35). Obesity is also associated with glucose intolerance and alterations in carbohydrate metabolism (36). Among important glucose metabolic enzymes are pyruvate kinase and phosphoenolpyruvate carboxykinase, both of which are highly sensitive to the nutritional and hormonal state of the animal (37,38).

Dietary polyunsaturated fats are potent inhibitors of hepatic fatty acid synthesis and triglyceride synthesis (39–41). It appears that alterations in dietary fat influence enzyme activity by up or down regulating gene transcription. Thus, it is important to understand the role of dietary fat in regulating energy metabolism and the lipid and carbohydrate metabolic enzymes at the genetic level in obese mice. The present study addresses the question of the effect of dietary fat composition on BAT energy metabolism and expression of other enzymes involved in lipid and carbohydrate metabolism in the liver.

MATERIALS AND METHODS

Animals and diets. Six-week-old male obese mice (ob/ob) and their lean litter mates (ob/+) or (+/+) (Jackson Laboratories, Bar Harbour, Maine) were caged in pairs and provided with water and diet *ad libitum*. The animal room was maintained at 24 ± 1°C with a 12-h light/12-h dark photo period. Diets contained the following essential nutrients per kg: 270 g high-casein protein, 200 g starch, 208 g glucose, 50 g nonnutritive cellulose, 10 g vitamin mix, 50 g mineral mix, 2.8 g choline chloride, 6.3 g inositol, and 2.5 g L-methionine. The lipid component of the diet was provided by mixing hydrogenated beef tallow, safflower oil, and linseed oil. Each diet contained 20% (w/w) fat. The amount of hydrogenated beef tallow, safflower oil, and linseed oil was altered to result in either a high (P/S = 1.0) or low (P/S = 0.25) polyunsaturated/saturated (P/S) fatty acid ratio (Table 1). At six weeks of age, half of the ob/ob and normal lean mice received the high P/S diet, and the remainder were fed the low P/S diet for an additional four weeks.

Mitochondrial preparation and analysis. Mice were killed by cervical dislocation, and BAT was pooled from interscapular, subscapular, and cervical regions. Mitochondria were isolated in a buffer consisting of 0.25 M sucrose, 5 mM NaTES, pH 7.2, and 1 mM EGTA from BAT pooled from two mice (42). Whole mitochondria were used immediately for measurement of purine nucleotide binding, and an inner mitochondrial membrane-matrix fraction was isolated for determination of cytochrome oxidase activity and analysis of fatty acid composition. The inner membrane-matrix fraction was prepared after incubation with digitonin, as described by Schnaitman and Greenawalt (43) and further purified by sucrose density gradient centrifugation (44). Whole and inner mitochondrial membrane protein content was determined spectrophotometrically (45). Activity of cytochrome oxidase in the inner membrane was determined by monitoring reduction of ferrocytochrome c (46). Purine nucleotide binding was assessed in whole mitochondria by measuring the specific binding of [^{3}H]GDP. Mitochondria were incubated for 10 min at 22°C in 100 mM [^{14}C] sucrose (6 kBq/ml; New England Nuclear), 10 μM [^{3}H]GDP (Na^{2+} salt, 10 kBq/ml; New England Nuclear, Sauga, Ontario, Canada), 20 mM TES, pH 7.1, 1 mM EDTA, and 5 μM rotenone (47).

TABLE 1
Fatty Acid Composition of Diets[a]

Diet (% w/w)	High P/S	Low P/S
14:0	1.8	3.4
16:0	16.9	23.8
18:0	25.9	43.2
18:1	8.9	6.5
18:2(6)	44.2	12.9
18:3(3)	1.3	1.2
Total saturated	43.5	78.60
Total polyunsaturated	45.6	20.60
P/S ratio	1.0	0.25

[a]Diet lipid was achieved by mixing fat in the following proportions: for polyunsaturated/saturated (P/S) 1.0—hydrogenated beef tallow 42%, safflower oil 56%, linseed oil 2%, for P/S 0.25—hydrogenated beef tallow 76%, safflower oil 22%, linseed oil 2%.

Lipid analysis. Total lipids were extracted from mitochondrial membranes in chloroform/methanol 2:1 (vol/vol) containing ethoxyquin as antioxidant followed by chloroform/methanol 7:1 (vol/vol) saturated with freshly prepared 28% aqueous ammonia. Extracts were then partitioned against 0.2 M KCL, dried, and then resuspended in chloroform/methanol 19:1 (vol/vol) and stored at –80°C under N_2. Phospholipid classes were separated on HP-K high-performance silica-gel plates (Whatman, Clifton, NJ) using chloroform/methanol/2-propanol/potassium chloride (0.25%)/triethylamine (20:9:25:6:18, vol/vol) as the solvent system (48). Individual phospholipid fatty acids were methylated using 14% (w/w) boron trifluoride in methanol. Fatty acid methyl esters were then separated by gas–liquid chromatography using a fused silica BP-20 capillary column (25 m × 0.25 mm i.d.; SGE, Ringwood, Australia). Analytical conditions used separated fatty acids from 12 to 24 carbons in length (49).

Quantitation of mRNA. Total RNA was extracted from liver samples using a guandinium-isothiocyanate method (50). RNA concentration was measured spectrophotometrically (A_{260}/A_{280}). A known amount of total RNA isolated was applied to nitrocellulose paper, and relative amounts of mRNA coding for fatty acid synthase (EC 2.3.1.85), acetyl-CoA carboxylase (EC 6.4.1.2), malic enzyme (EC 1.1.1.40), pyruvate kinase (EC 2.7.1.40), and phosphoenolpyruvate carboxykinase (EC 4.1.1.32) were determined by slot-blot hybridization with specific ^{32}P-labeled cDNA probes. The probes, donated by D.W. Back (Queen's University,

TABLE 2
Body Weights of ob/ob and Normal Lean Control Mice Fed a High or Low P/S Ratio Diet

	P/S	Lean	Obese
Initial weight (g)	1.0	18.1 ± 1.3	36.0 ± 2.6
Six weeks	0.25	17.9 ± 1.2	35.3 ± 2.5
Final weight (g)	1.0	22.1 ± 1.2	53.5 ± 2.7[a]
	0.25	20.7 ± 1.6	48.9 ± 2.7

[a]Significantly different from obese mice fed the P/S 0.25 diet, P < 0.05, n = 16. Six-week-old obese and lean mice were fed experimental diets for four additional seeks. See Table 1 for abbreviations.

Kingston, Ontario, Canada), were labeled by the random primer method (Prima-A-Gene labeling system; Promega). Hybridization was carried out in a shaking water bath for 36 h at 42°C. The hybridization solution contained 40% formamide, 4*x* SSPE buffer, 10% dextran sulphate, 5*x* Denhardt's solution, 0.2% SDS, and 100 mg/mL calf thymus DNA. After hybridization, the membranes were washed in 2*x* SSC, 0.1% SDS, 0.1*x* SSC, 0.1% SDS, 0.1*x* SSC, and 0.1% SDS at 50°C for 30 min. Membranes were air-dried, and slots were cut out and counted in a scintillation counter for ^{32}P. Nonspecific binding was elevated using pBR322 plasmid.

Enzyme assays. Activity of fatty acid synthase was measured following the method of Nepokroeff *et al.* (51). A unit of enzyme activity represents the synthesis of 1 nmole of palmitic acid per min. Malic enzyme was determined using the method of Hsu and Lardy (52). The rate of NADPH formation was proportional to enzyme concentration up to a change of 0.2 optical density units per min at 340 mμ. The method of Inoue and Lowenstein (53) was followed to determine acetyl-CoA carboxylase. One unit of enzyme activity was equal to 1 μmole of malonyl-CoA formed at 37°C per min. Pyruvate kinase and phosphoenolpyruvate carboxykinase were assayed following the method of Imamura and Tanaka (54) and Petrescu et al. (55), respectively. For pyruvate kinase, 1 unit of enzyme was defined as the amount catalyzing the formation of 1 μmole of pyruvate/min. Phosphoenolpyruvate carboxykinase activity was expressed as nmoles NADH oxidized/min/mg protein.

Statistical analysis. The effect of diet treatment and physiological state on enzyme activities, GDP binding, and mRNA levels were compared by analysis of variance procedures. Values are group means ± SEM for n = 6. Treatments without a common superscript are significantly different ($P < 0.01$).

RESULTS

At the commencement of dietary treatment (age 42 d), obesity was evident in the ob/ob genotype. After four weeks of feeding (age 70 d), the body weight of the obese mice that had been fed the high P/S diet was significantly greater than the obese mice that had been fed the low P/S diet (Table 2). There was no significant effect of diet on the body weight of the lean animals. While obese animals that had been fed the high P/S diet gained more weight than those on the low P/S diet, it should be noted that total energy intake was not measured, and therefore we cannot eliminate the possibility of minor differences in fat absorption.

Cytochrome oxidase activity. The activity of cytochrome oxidase did not differ by diet or genotype (Fig. 1).

Purine nucleotide binding. The specific binding of GDP to BAT mitochondria was influenced by genotype and dietary lipid. GDP binding was significantly lower ($P < 0.01$) in ob/ob mice that had been fed high or low P/S diet, compared to normal lean controls that had been fed the same diets (Fig. 1). The ratio of GDP binding was the same between obese and lean animals that had been fed the same diet. GDP binding in both obese and lean animals fed a low P/S diet was reduced, but not significantly, compared to animals that had been fed a high P/S diet (Fig. 1). GDP binding, however, was not different between obese mice that had been fed a high P/S diet compared to normal lean mice fed a low P/S diet.

Inner mitochondrial membrane fatty acid composition Fatty acid composition of BAT inner mitochondrial membrane from the ob/ob mouse is influenced by genotype and by type of dietary fatty acids consumed. Obese animals that had been fed a low P/S diet exhibited lower membrane levels of total saturates in the phosphatidylcholine (PC) (Table 3), phosphatidylethanolamine (PE) (Table 4), and cardiolipin fractions (Table 5). For the PC and PE fractions, reduction in total saturates was due primarily to lower levels of 18:0, whereas in cardiolipin, both 16:0 and 18:0 were significantly lower (data not illustrated). Similar trends were observed in animals that had been fed the high P/S diet (i.e., a decrease in

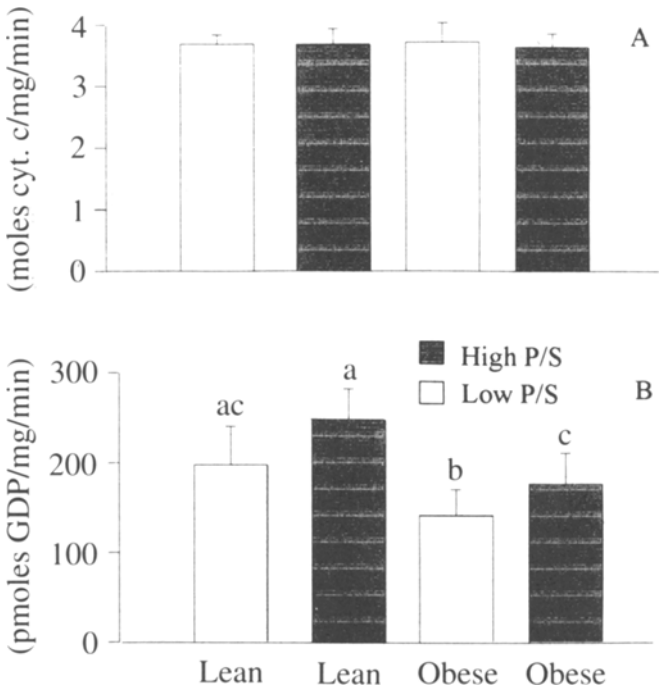


FIG. 1. Cytochrome oxidase activity (A) and GDP binding in brown adipose tissue mitochondria (B) from obese and normal lean mice fed a high or low P/S diet. Results are expressed as means ± SEM, n = 8. Bars that do not share a common superscript are significantly different ($P < 0.05$).

levels of total saturated fatty acids), with the exception that in the cardiolipin fraction total saturates were lower, but not significantly. Lower levels of 18:0 in obese animals accounted for the lower total levels of saturates.

In all three of the phospholipid fractions examined, total levels of monounsaturated fatty acids were higher in obese animals, regardless of diet fed (Tables 3–5). All the major monounsaturated fatty acids increased in obese animals: 16:1, 18:1n-9, and 18:1n-7. In the obese state, the inner mitochondrial membrane accumulated linoleic acid (18:2n-6) in PC and PE in animals that had been fed the high P/S diet. Levels of 18:2n-6 were lower in cardiolipin and in PE in animals on the low P/S diet. At the same time, levels of arachidonic acid (20:4n-6) declined in PC, but increased in PE and cardiolipin. In contrast, 20:3n-6 accumulated in all phospholipid fractions examined, with the result that the ratio of 20:3n-6/18:2n-6 (Δ6-desaturase index) increased in all three phospholipid fractions. Relatively high levels of 20:4n-6 in the PE fraction, coupled with a small increase in 20:3n-6 in the obese state, suggests an apparent inhibition of Δ5-desaturase activity. Similar decreases in the Δ5-desaturase index were observed in the PC and cardiolipin fractions. While significant accumulation of 18:2n-6 occurred in the membrane, the total level of n-6 polyunsaturated fatty acids did not increase in obese animals that had been fed either diet, compared to their lean controls.

In the same genotype, feeding a diet high in n-6 polyunsaturated fatty acids (P/S = 1.0) influenced the fatty acid composition of PC, PE, and cardiolipin relative to a saturated fatty acid diet (P/S = 0.25). For the PC fraction, total levels of saturated fatty acids were lower in lean animals that had been fed the high P/S diet, with 18:0 being reduced the most.

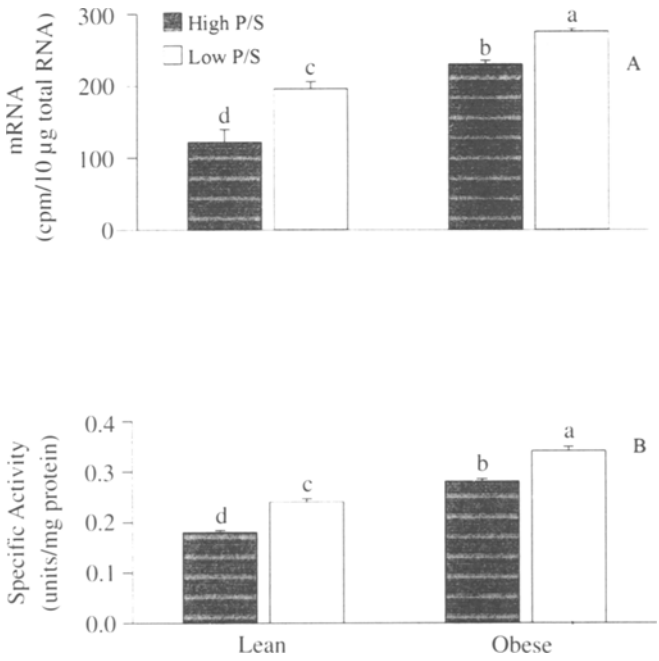


FIG. 2. Hepatic fatty acid synthase mRNA and specific activity. Values are means ± SEM, n = 6. Bars without a common superscript are significantly different ($P < 0.01$).

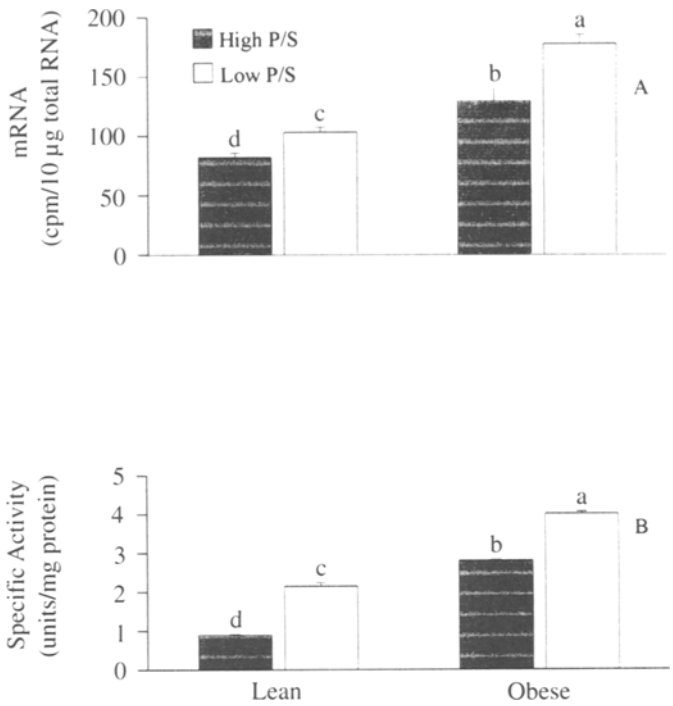


FIG. 3. Relative abundance of hepatic malic enzyme mRNA and malic enzyme specific activity. Values are means ± SEM, n = 6. Bars without a common superscript are significantly different.

Similar reductions in 18:0 in the PE and cardiolipin fractions resulted in an overall decrease in total saturation of these phospholipids. The influence of the high n-6 diet did not appear as marked in obese animals. While this trend was toward a lowering of total saturates, differences were not significant. The influence of diet was greatest on relative amounts of monounsaturated fatty acids. In all phospholipid fractions, total levels of monounsaturated fatty acids decreased in animals fed the P/S = 1.0 diet, compared to the same genotype on the P/S = 0.25 diet. With a few exceptions, notably in the PE fraction, 16:1, 18:1n-9, and 18:1n-7 were all lower in animals that had been fed the high P/S diet.

Feeding the high n-6 diet had the effect of elevating membrane levels of total n-6 fatty acids in both lean and obese animals (Tables 3–5). In all cases, 18:2n-6 accumulated in all the phospholipid fractions; there was no change in membrane levels of 20:4n-6 in PC and PE, but there was an increase of 20:4n-6 in cardiolipin. The Δ6-desaturase index was not affected by diet in the PC fraction from lean animals, the PE fraction from obese animals, or cardiolipin from both lean and obese animals, but decreased in all other animals fed the P/S = 1.0 diet. The Δ5-desaturase index was lower in PC from lean and obese animals that had been fed the high P/S diet, but higher in the cardiolipin fraction from the same animals. In the PE fraction, the Δ5-desaturase index was higher in lean animals fed the high P/S diet compared to the low P/S diet and was highest in obese animals.

Membrane levels of n-3 fatty acids were lower in all phospholipid fractions from animals that had been fed the high P/S diet resulting in significant increases in the n-6/n-3 ratio in all the phospholipid classes.

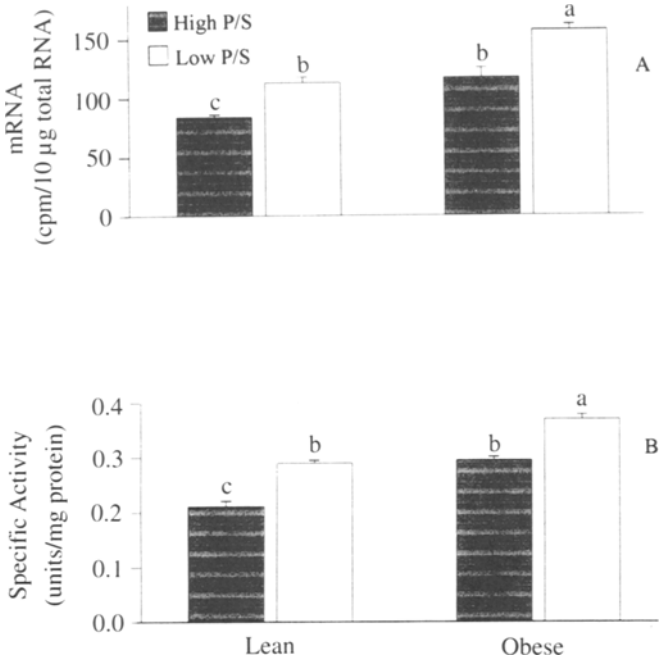


FIG. 4. Hepatic acetyl-CoA carboxylase mRNA level and specific activity. Values are means ± SEM, n = 6. Bars without a common superscript are significantly different.

Effect of diet and obesity on enzymes of lipogenesis. Combined data from six animals per group for the mRNA levels and enzyme activities of fatty acid synthase, acetyl-CoA carboxylase, and malic enzyme are illustrated (Figs. 2–4).

Hepatic fatty acid synthase and malic enzyme mRNA levels were significantly elevated in ob/ob mice, compared to lean mice (Fig. 2A and 3A). Fatty acid synthase and malic enzyme activity also showed a significant increase in ob/ob mice compared to lean mice (Figs. 2B and 3B). Dietary polyunsaturated fat (P/S = 1.0) depressed both fatty acid synthase and malic enzyme activity in both lean and obese mice. Suppression of fatty acid synthase and malic enzyme activity by dietary polyunsaturated fats is associated with a decrease in fatty acid synthase mRNA and malic enzyme mRNA abundance (Figs. 2A and 3A).

mRNA level and enzyme activity of acetyl-CoA carboxylase increased significantly in ob/ob mice, as compared to lean mice (Fig. 4A). Increase in mRNA level was concomitant with an increase in enzyme activity for acetyl-CoA carboxylase in obese mice compared to lean mice (Fig. 4B). Lower levels of dietary saturated fatty acid and high levels of dietary polyunsaturated fat seemed to depress transcription of acetyl-CoA carboxylase and enzyme activity in both lean and obese mice (Figs. 4A and 4B).

Effect of diet and obesity on carbohydrate metabolic enzymes. Hepatic pyruvate kinase mRNA level increased in ob/ob mice, compared to lean mice (Fig. 5A). Pyruvate kinase enzyme activity also increased in ob/ob mice, as compared to lean mice (Fig. 5B). Dietary fat composition had no effect on pyruvate kinase mRNA level or enzyme activity in either lean or obese mice, compared to mice that had been fed a diet high in saturated fats (Figs. 5A and 5B).

There was no change in phosphoenolpyruvate carboxykinase specific activity (mean ± SEM) and mRNA level (mean ± SEM level) in lean and obese mice (Figs. 6A and 6B) fed either the high (P/S = 1.0) or low (PS = 0.25) P/S diets.

DISCUSSION

In the present study, feeding a high P/S diet compared to a low-P/S diet increased GDP binding in both lean and obese mice, indicating that a high-fat diet appears to influence diet-induced thermogenesis in obese (ob/ob) mouse. Earlier studies suggest that the extent to which energy expenditure is enhanced may be dependent on the fatty acid composition of the diet (22). In particular, a high-fat diet rich in polyunsaturated fatty acids is more effective at stimulating thermogenic activity in BAT than is a diet rich in saturated fatty acids. The mechanism by which fatty acids regulate BAT thermogenesis is unclear but may be the consequence of direct stimulation of the proton conductance pathway or modulation of sympathetic regulation. Another possibility suggested by others (56,57) and by this study is that effective proton conductance of the inner mitochondrial membrane is determined in part by the lipid environment of the pathway. The obese state may foster a lower proton conductance pathway and consequently an impairment of thermogenesis.

In the present study, a clear pattern of alterations in fatty acid composition in the BAT inner mitochondrial membrane is also apparent. Inner mitochondrial membranes from obese animals are less saturated, but higher in monounsaturated fatty acids. The fact that the level of 18:0 is lower in obese animals with a corresponding increase in 16:1n-7 and 18:1n-9 suggests

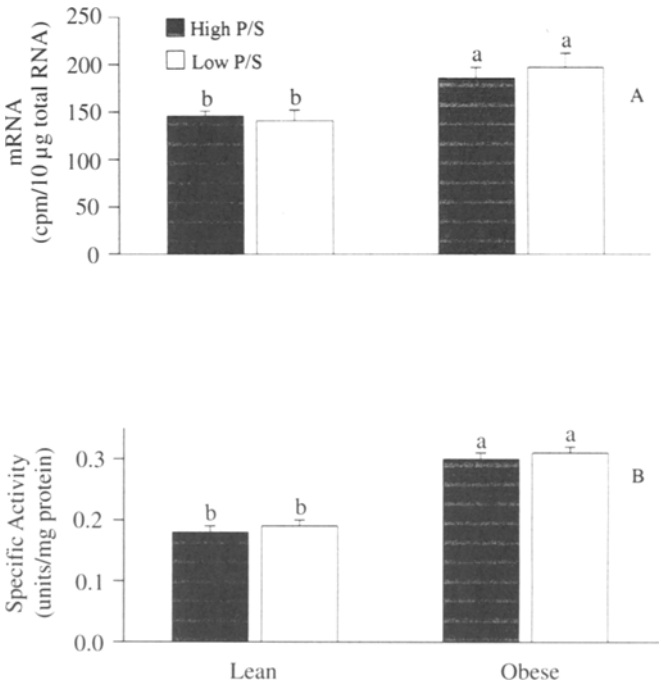


FIG. 5. Relative abundance of hepatic pyruvate kinase mRNA and specific activity of pyruvate kinase. Values are means ± SEM, n = 6. Bars without a common superscript are significantly different.

TABLE 3
Fatty Acid Composition of Phosphatidylcholine from Brown Adipose Tissue Inner Mitochondrial Membrane from Lean (ob/-) and Obese (ob/ob) Mice Fed High or Low P/S Diet[a]

	P/S 0.25		P/S 1.0	
Fatty acid (mol %)	Lean	Obese	Lean	Obese
ΣSAT[b]	54.5 ± 1.4	46.8 ± 1.3[d]	49.5 ± 1.8[e]	45.0 ± 0.7[d]
ΣMONO[c]	16.8 ± 0.7	24.2 ± 1.8[d]	10.6 ± 0.9[e]	19.1 ± 0.7[d,e]
18:2n-6	16.0 ± 1.0	18.2 ± 1.1[d]	19.8 ± 0.9[e]	28.7 ± 1.4[d,e]
20:3n-6	0.3 ± 0.1	0.9 ± 0.2[d]	0.4 ± 0.2[e]	1.1 ± 0.2[d]
20:4n-6	5.3 ± 0.3	3.7 ± 0.2[d]	5.0 ± 0.2	3.5 ± 0.3[d]
Δ6-desaturase index (20:3n-6/18:2n-6.100)	1.9	4.9[d]	1.9	3.8[d,e]
Δ5-desaturase index (20:4n-6/20:3n-6)	16.6	4.1[d]	13.5[e]	3.2[d,e]

[a]Values represent mean ± SEM for n = 6 animals. See Table 1 for other abbreviations.
[b]ΣSAT, sum of saturated fatty acids.
[c]ΣMONO, sum of monounsaturated fatty acids.
[d]Significantly different from lean controls fed the same diet; $P < 0.05$.
[e]Significantly different from same genotype fed the P/S 0.25 diet; $P < 0.05$.

a stimulation or enhanced induction of the Δ9-desaturase pathway. Thus it would appear that the thermogenic response of BAT can be modulated by dietary lipid levels independent of the state of obesity. Similar increases in monounsaturated fatty acids have been observed in ob/ob mouse hepatic plasma membranes (58), total liver phospholipids, and epididymal fat pad triglycerides (29), and obese (fa/fa) rat serum, from the kidney and heart (59). In contrast to monounsaturated fatty acids, the majority of tissues from obese animals appeared to have reduced levels of linoleic acid (58–59). One significant exception was an increase in 18:2n-6 in total heart phospholipids in the obese Zucker rat (59). In the present study, 18:2n-6 levels were also higher in PC from obese mice, regardless of diet, and in cardiolipin from obese mice fed a high P/S diet. The observation that the Δ6-desaturase index was higher for all three phospholipid fractions in obese animals suggests that a higher proportion of 18:2n-6 is desaturated and elongated to other polyunsaturated fatty acids in the obese state. The apparent reduction in 18:2n-6 levels in PE and cardiolipin was accompanied by a reduction in membrane arachidonic acid, albeit to a lesser degree. The net result was that the ratio of 20:4/20:3 indicated that Δ5-desaturase was inhibited, thus reducing the further desaturation and elongation of 18:2n-6 to longer-chain polyunsaturates. This too appeared to be common to other tissues examined in genetically obese animals in that lower Δ5-desaturase activity has been observed in ob/ob mouse total liver phospholipids (29) and several tissues in the obese Zucker rat (59). It is worth noting that, in the other studies reported, only total lipid or total phospholipid analysis was undertaken, and differences in individual phospholipid class composition were not measured.

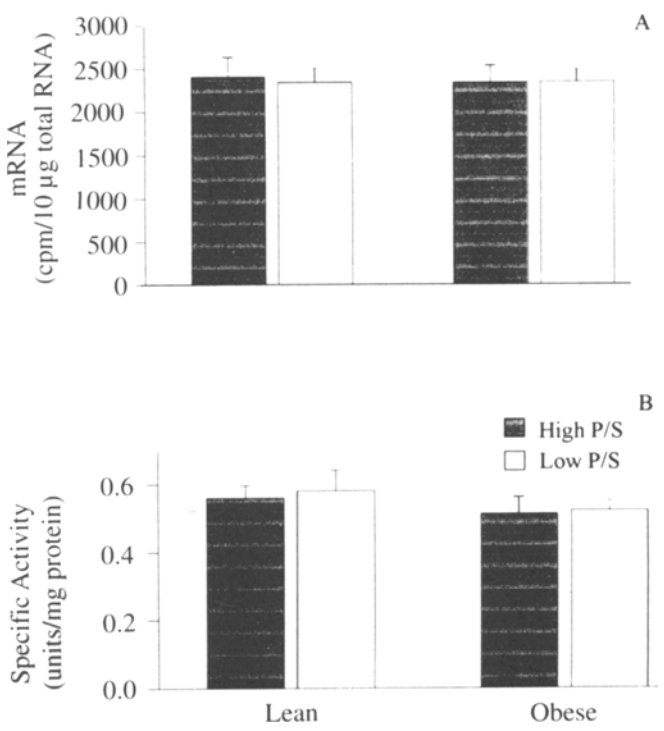


FIG. 6. Relative abundance of phosphoenolpyruvate carboxykinase mRNA and specific activity. Values are means ± SEM, n = 6.

Unlike liver phospholipids (29), metabolism of n-3 fatty acids appeared to be relatively unaffected in the BAT inner mitochondrial membrane of obese mice. Activity of cytochrome oxidase did not differ by diet or genotype, indicating that oxidative capacity of mitochondria was not altered in the ob/ob genotype, nor influenced by dietary lipid treatment under the conditions described. This is not unexpected, as all three phospholipid classes examined had low levels of total n-3 fatty acids and only trace amounts of 18:3n-3. Since 18:2n-6 and 18:3n-3 compete as substrates for Δ6-desaturase, reduction of total n-3 fatty acids in both lean and obese animals on the high-P/S diet reflects domination of the desaturation pathway by 18:2 as the result of high dietary levels. The result is that the ratio of n-6/n-3 fatty acids increased in both lean and obese animals that had been fed the high P/S diet. These observations suggest that at least by the end of a four-week feeding regimen, a diet high in polyunsaturated fatty acids may partially ameliorate the defect in activity of the proton conductance pathway in the ob/ob mouse.

Relating changes in membrane fatty acid composition to proton conductance in genetic obesity is not easily achieved.

TABLE 4
Fatty Acid Composition of Phosphatidylethanolamine from Brown Adipose Tissue Inner Mitochondrial Membrane from Lean (ob/-) and Obese (ob/ob) Mice Fed High or Low P/S/ Diet[a]

	P/S 0.25		P/S 1.0	
Fatty acid (mol %)	Lean	Obese	Lean	Obese
ΣSAT[b]	42.5 ± 1.0	31.5 ± 1.1[d]	39.0 ± 0.9[e]	32.8 ± 1.5[d]
ΣMONO[c]	13.0 ± 0.9	18.3 ± 1.5[d]	12.1 ± 1.4	16.9 ± 1.3[d,e]
18:2n-6	10.1 ± 0.9	8.3 ± 0.7[d]	12.1 ± 1.0[e]	16.9 ± 1.2[d,e]
20:3n-6	0.4 ± 0.2	0.5 ± 0.2	0.3 ± 0.1	0.6 ± 0.2[d]
20:4n-6	29.5 ± 1.1	32.9 ± 1.4[d]	28.6 ± 1.1	32.6 ± 1.6[d]
Δ6-desaturase index (20:3n-6/18:2n-6.100)	3.9	6.0[d]	2.2[e]	6.1[d]
Δ5-desaturase index (20:4n-6/20:3n-6)	74.0	65.8[d]	95.0[e]	54.3[d,e]

[a]Values represent mean ± SEM for n = 6 animals. See Table 1 for other abbreviations.
[b]ΣSAT, sum of saturated fatty acids.
[c]ΣMONO, sum of monounsaturated fatty acids.
[d]Significantly different from lean controls fed the same diet; $P < 0.05$.
[e]Significantly different from same genotype fed the P/S-0.25 diet; $P < 0.05$.

There is some controversy as to the physiological significance of GDP binding measurements, with recent evidence suggesting that binding reflects activity rather than purported alterations in the amount of uncoupling protein (60). In cold-exposed rats, GDP-dependent changes in proton conductance appeared to be associated with functional changes in the pathway rather than the amount of uncoupling protein or masking of its active sites (61). If this is indeed the case, the present study confirms that in the obese state thermogenesis is depressed as a consequence of a decrease in activity of the proton conductance pathway. It also confirms that activity of the pathway can potentially be modified by diet fat and perhaps by fatty acid constituents of the inner mitochondrial membrane. GDP-dependent proton conductance was enhanced in rats deficient in EFA, both at thermoneutrality and when cold adapted (62). In the presence of GDP, a significant proportion of GDP-independent proton conductance can be measured in EFA-deficient rats, indicating existence of a basal conductance, that may be influenced by membrane fatty acid composition. At present, it is difficult to explain how an EFA-deficient diet, which undoubtedly depletes membrane 18:2n-6, enhances proton conductance whereas in the present study a high 18:2 diet also results in an apparent increase in proton conductance. Perhaps diet-induced changes in the phospholipid class content are also involved (63).

In summary, the present study demonstrates that metabolism of monounsaturated and n-6 polyunsaturated fatty acids are altered in the inner mitochondrial membrane of the ob/ob mouse. Apparent transitions in Δ5-desaturase activity and enhanced Δ6-desaturase activity serves to change the fatty acid profile of PC, PE, and cardiolipin in inner mitochondrial membranes of obese animals. Feeding a diet high in linoleic acid accentuates these changes. While we have not determined to what extent the impact of altered lipid environment on the proton conductance pathway contributes to the overall depression of thermogenesis in the ob/ob mouse, it is clear that activity of proton channels can be modulated by dietary fatty acids.

A defect in regulation of fatty acid synthesis was postulated in obesity many years ago (64). Since then, high-fat diets (>30% cals from fat) have been shown to inhibit lipogenesis. Dietary polyunsaturated fats are potent inhibitors of hepatic fatty acid and triglyceride synthesis. The objective of this study was to determine if the effect of dietary polyunsaturated fats on hepatic gene transcription for lipogenesis and the conversion of carbohydrate to lipid is different for the obese state when compared to the lean state. This comparison was made at a dietary fat level (40% cals from fat) where a near-maximal level of inhibition of *de novo* lipogenesis from carbohydrate would be expected. Data from this study demonstrate that higher activities of fatty acid synthase, acetyl-CoA carboxylase, malic enzyme, and pyruvate kinase in the liver of obese mice is due to stimulation of gene transcription measured at higher tissue mRNA levels. In the obese state, these results are consistent with a major role for increased *de novo* fatty acid synthesis in the pathogenesis of obesity in ob/ob mice. The close correlation between mRNA levels and specific enzyme activity suggests that regulation of fatty acid synthase, malic enzyme, acetyl-CoA carboxylase, and pyruvate kinase was at the pretranslational level, mainly at the level of gene transcription.

The findings suggesting that low dietary saturated fat and high dietary polyunsaturated fat suppressed expression of fatty acid synthase are consistent with those recently observed (65). This high dietary polyunsaturated fat ratio (P/S = 1.0) also suppressed mRNA levels and enzyme activities for malic enzyme and acetyl-CoA carboxylase in both lean and obese mice; however, mRNA level and enzyme specific activity for pyruvate kinase and phosphoenolpyruvate carboxykinase were not affected by dietary polyunsaturated fats (P/S = 1.0). Fatty acid synthase revealed maximum suppression by dietary polyunsaturated fats, compared to malic enzyme and acetyl-CoA carboxylase. Decrease in liver fatty acid synthase mRNA level was more pronounced in lean mice, as compared to obese mice, which reveals that obese mice are resistant to polyunsaturated fatty acid feedback control of gene expression.

TABLE 5
Fatty Acid Composition of Cardiolipin from Brown Adipose Tissue Inner Mitochondrial Membrane from Lean (ob/-) and Obese (ob/ob) Mice Fed High or Low P/S Diet[a]

	P/S 0.25		P/S 1.0	
Fatty acid (mol %)	Lean	Obese	Lean	Obese
ΣSAT[b]	33.2 ± 1.1	29.4 ± 1.2[d]	29.5 ± 1.0[e]	27.9 ± 0.9[d]
ΣMONO[c]	21.8 ± 0.8	25.4 ± 0.9[d]	19.2 ± 0.9[e]	22.7 ± 0.8[d,e]
18:2n-6	36.6 ± 2.0	34.7 ± 1.8	42.2 ± 2.5[e]	39.2 ± 1.9[d,e]
20:3n-6	1.0 ± 0.2	1.6 ± 0.2[d]	1.2 ± 0.1	1.7 ± 0.2[d]
20:4n-6	2.5 ± 0.1	3.3 ± 0.2[d]	3.7 ± 0.3[e]	4.5 ± 0.2[d,e]
Δ6-desaturase index (20:3n-6/18:2n-6.100)	2.7	4.6[e]	2.8	4.3[d]
Δ5-desaturase index (20:4n-6/20:3n-6)	2.5	2.1	3.1[e]	2.6[d,e]

[a]Values represent mean ± SEM for n = 6 animals. See Table 1 for other abbreviations.
[b]ΣSAT, sum of saturated fatty acids.
[c]ΣMONO, sum of monounsaturated fatty acids.
[d]Significantly different from lean controls fed the same diet; $P < 0.05$.
[e]Significantly different from same genotype fed the P/S-0.25 diet; $P < 0.05$.

Mechanisms by which dietary polyunsaturated fats inhibit fatty acid synthase, acetyl-CoA carboxylase, and malic enzyme gene expression has been difficult to elucidate. Induction of fatty acid synthase is explicable if polyunsaturated fatty acid or a metabolic end-product directly regulates fatty acid synthase mRNA production either by controlling transcription, RNA processing, or mRNA stability (65). Clarke and Jump (66) proposed that n-6/n-3 fatty acids, which inhibit fatty acid synthase and S14 gene transcription, are transported through the plasma membrane and subsequently bind to the cytosolic fatty acid binding protein (FABP). They further hypothesized that the FABP shuttles the 18-carbon fatty acids to the Δ6-desaturase and subsequently carries fatty acid products to the nucleus, where the cytosolic FABP is transferred to a specific nuclear FABP. This nuclear FABP binds to a specific *cis*-acting element, which operates to govern gene transcription.

Suppression of mRNA level and enzyme activities by polyunsaturated fatty acids was highly specific for lipogenic enzymes and was not observed for pyruvate kinase or phosphoenolpyruvate carboxykinase. The findings in the present study for pyruvate kinase are similar to those of Toussant *et al.* (67), who showed that activities of pyruvate kinase were altered by dietary linoleate. Taken together, results of this study demonstrate that expression of genes for fatty acid synthase, malic enzyme, acetyl-CoA carboxylase, and pyruvate kinase is higher in the liver of the ob/ob mouse, with no effect on phosphoenolpyruvate carboxykinase gene expression. The overexpression of these enzymes appears to be an early feature of the onset of obesity.

ACKNOWLEDGMENTS

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Dietary Marine Lipids Suppress Continuous Expression of Interleukin-1β Gene Transcription[1]

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ABSTRACT: n-3 Polyunsaturated fatty acids abundant in marine lipids suppress certain inflammatory and immune reactions, and dietary marine lipid supplements have antiinflammatory effects in experimental and human autoimmune disease. Previous work by other investigators demonstrated that dietary marine lipid supplements suppressed production of cytokines from stimulated human peripheral blood mononuclear cells *ex vivo*. The present study further documents the ability of n-3 fatty acids to inhibit cytokine formation, and in part defines the mechanism of the inhibition of production of interleukin-1β (IL-1β) by dietary n-3 fatty acid. Female BALB/c mice were each fed a fat-free balanced diet to which was added either a refined fish oil (FO) preparation as a source of n-3 fatty acid, or beef tallow (BT), which consisted primarily of saturated and monoenoic fatty acids. After ingesting the experimental diets for periods ranging from 3 to 12 wk, spleen cell preparations were stimulated *ex vivo* with either lipopolysaccharide (LPS) or phorbol 12-myristate 13-acetate (PMA), and proIL-1β mRNA (IL-1β mRNA) was measured by northern analysis. Levels of IL-1β mRNA in both LPS- and PMA-stimulated cells from BT-fed mice were elevated to a greater extent than in cells from FO-fed mice, at most concentrations of LPS and PMA. Stability of LPS-stimulated mRNA levels after actinomycin D was similar for BT and FO groups, indicating that lower levels of IL-1 mRNA with FO groups was related to suppressed IL-1 gene transcription and not due to accelerated transcript degradation. Nuclear run-on transcription assays revealed a more transient expression of the IL-1β gene in LPS-stimulated spleen cells from FO-fed mice compared to cells from BT-fed mice. We conclude that dietary marine lipids reduce transient expression of the IL-1β gene in stimulated splenic monocytic cells. Preliminary results from nuclear run-on transcription assays indicate that n-3 fatty acids may not change the initial rate of gene transcription but may promote more rapid shutting down of transcription of this gene after induction than do alternative lipids.

Lipids 31, S-23–S-31 (1996).

Dietary n-6 and n-3 polyunsaturated fatty acids are essential for normal health in humans and other mammalian species. A large body of evidence from epidemiologic and experimental studies has also documented that the relative quantities of n-6 and n-3 fatty acids can alter a variety of cell functions and disease states (1–3). Dietary n-3 fatty acids are incorporated into tissue phospholipids, accompanied by replacement of n-6 fatty acids, including arachidonic acid. These changes are associated with reductions in the capacity of cells to produce eicosanoids, and it has been postulated that reduced production of prostaglandins and leukotrienes by cells enriched in n-3 fatty acids may contribute to their antiinflammatory effects. It is also clear, however, that alterations in eicosanoid formation cannot account for all the biological effects of n-3 fatty acids (4). In addition to inhibition of eicosanoid production, the presence of elevated levels of n-3 fatty acids may suppress cytokine formation. A study of human volunteers demonstrated that a dietary supplement of marine oil for 6 w significantly reduced quantities of interleukin-1α (IL-1α), IL-1β, and tumor necrosis factor-α (TNF-α) produced by lipo-polysaccharide (LPS)-stimulated peripheral blood mononuclear cells *ex vivo*, as measured by radioimmunoassay (5). An additional study of patients with rheumatoid arthritis ingesting a dietary supplement of fish oil (FO) found suppression of IL-1 production by LPS-stimulated peripheral blood mono-nuclear cells, compared to control patients ingesting an olive oil supplement (6). These reports raise the possibility that suppression of cytokine expression by n-3 fatty acids may contribute to antiinflammatory effects of these lipids.

Previous studies demonstrated that dietary marine lipids markedly reduced severity of the spontaneous autoimmune glomerulonephritis in murine lupus strains, which are models for the human disease, systemic lupus erythematosus (7–10). Subsequent controlled clinical trials documented modest but significant antiinflammatory effects of dietary marine lipid supplements in patients with rheumatoid arthritis, and one study reported beneficial effects of dietary marine lipids in patients with systemic lupus erythematosus (6,11–14). Mechanisms of these well-documented antiinflammatory effects of n-3 fatty acids are unclear. n-3 Fatty acids were shown to suppress formation of eicosanoids, including leukotriene B4 and other 5-lipoxygenase

[1]The IL-1β cDNA probe detects proIL-1β mRNA, which is referred to as IL-1β mRNA throughout the paper for convenience.

*To whom correspondence should be addressed at the Arthritis Unit, Bulfinch 165, Massachusetts General Hospital, 32 Fruit St., Boston, MA 02114. Abbreviations: BT, beef tallow; FO, fish oil; IL-1, interleukin-1; LPS, lipopolysaccharide; LT, leukotriene, PG, prostaglandin; PMA, phorbol 12-myristate 13-acetate; TNF-α, tumor necrosis factor-α.

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products (6,15). Cyclooxygenase products may also be inhibited by n-3 fatty acids (4), but this effect seems unlikely to account for beneficial effects of n-3 fatty acids on murine lupus, since a cyclooxygenase inhibitor did not alter the course of autoimmune disease in one of these strains (16).

Several lines of evidence indicate that cytokines play an important role in the pathogenesis of inflammatory reactions (17–19). Elevated levels of mRNA for IL-1β and TNF-α are present in kidneys of mice with autoimmune glomerulonephritis, and administration of recombinant IL-1α and TNF-α may aggravate murine autoimmune disease under certain conditions (20,21). Synovial tissues from patients with rheumatoid arthritis contain IL-1, TNF-α, and other cytokines, and these and other observations led to the conclusion that these cytokines have an important role in the pathogenesis of this disease (18,22).

To clarify mechanisms by which n-3 fatty acids suppress cytokine production, we have investigated the effects of a dietary marine lipid on the levels of IL-1β mRNA in murine spleen cells that have been stimulated to produce cytokines *ex vivo*. Results of these studies demonstrate that dietary n-3 fatty acids reduce steady-state IL-1β mRNA levels in isolated spleen cells that have been stimulated with either LPS or phorbol 12-myristate 13-acetate (PMA). Experiments with actinomycin D–treated cells as well as nuclear run-on transcription assays demonstrated that dietary n-3 fatty acids reduce IL-1β production by depressing the total amount of gene transcription. Furthermore, preliminary examination of nuclear run-on kinetics suggests a mechanism for reduced IL-1β gene transcription involving an effect targeted not at the activation of gene transcription, but alternatively at the distal regulatory repression that generates the transient transcriptional response usually observed for this gene. Consequently, n-3 fatty acids, in contrast to alternative fatty acids, could result in more efficient activation of the transcriptional clamp previously described (23).

METHODS

Diets. Experimental diets consisted of a fat-free powder (85 wt%) to which was added one of two lipid preparations (15%). The fat-free powder (ICN Nutritional Biochemicals, Cleveland, OH) provided a balanced diet consisting of casein 21%, cellulose 15.6%, sucrose 58.5%, and a balanced salt mixture and essential vitamins, including 50 mg/kg of α-tocopherol. Lipids were stabilized with 0.02% *t*-butylhydroquinone. Female BALB/c mice were fed with experimental diets beginning at age 2 mo. Diets were given *ad libitum*, except for brief restrictions occasionally to maintain mean weights of each dietary group within 5% of each other. Fatty acid composition of dietary lipids has been published elsewhere (24). Beef tallow (BT) (ICN Nutritional Biochemicals) primarily consisted of saturated (48%) and monoenoic (47%) fatty acids, with 4.7% n-6 fatty acid in the form of linoleic acid and essentially no (<1%) n-3 fatty acids. The FO preparation (Nissui 28; Nippon Suisan Kaisha, Ltd., Tokyo) contained 55% n-3 fatty acid, 3.5% n-6 fatty acid, with the remainder (40%) as saturated and monoenoic fatty acids.

Cell preparation and stimulation. Groups of mice were sacrificed by cervical dislocation after feeding with experimental diets for varying time intervals. Spleens were rapidly removed and minced, and erythrocytes were hemolyzed with NH_4Cl. Spleen cells were washed three times with RPMI 1640 (Cellgro; Mediatech Inc., Herndon, VA) and filtered through fine mesh gauze. Cell viability was over 95%, based on trypan blue dye exclusion. Spleen cells (10^8) were cultured in 5 mL RPMI 1640 medium with or without varying doses of LPS (*E. coli* 055:B5, Sigma Chemical Co., St. Louis, MO) or PMA (Sigma Chemical Co.) for 4 h or other time intervals as indicated. Analysis of a representative spleen cell preparation fluorescence-activated cell sorter utilizing monoclonal antibodies to immunoglobulin G and Thy-1 revealed 38% Ig^+ cells, 29% Thy-1^+ cells. The small percentage of monocytes could not be determined accurately by the cell sorter using an anti-Mac-1, but histologic analyses of Wright–Giemsa-stained smears revealed 3–5% monocytes, with no differences in percent monocytes between FO and BT groups in two experiments. Others have reported that percentages of lymphocytes and monocytes in murine spleen cell preparations similar to ours were not altered by dietary n-3 fatty acid (25).

Preparation of mRNA and northern blot analysis. Total cellular RNA was prepared by guanidinium thiocyanate lysis (26). Gel electrophoresis of RNA was carried out using 1% agarose gels containing 6% formaldehyde, loading the gels with 10 mg RNA/lane (27). Ethidium bromide was added to the loading buffer, and the 28S and 18S ribosomal RNA bands were located with ultraviolet light. RNA was then transferred to nitrocellulose filters (Schleicher & Schull, Keene, NH). Prehybridization, hybridization, and autoradiography were performed according to standard procedures (28). The murine IL-1β probe was a 1600-base pair fragment of the full-length cDNA (29). The β-actin probe (provided by Dr. Mary Goldring) was a 700-base pair fragment of the full-length cDNA (30). cDNA Probes were labeled with [α-^{32}P]dCTP using a random primer method (31).

Nuclear isolation and transcriptional run-on assay. For run-on transcription experiments, spleen cells were incubated with 1 ng/mL LPS for 0 to 3 h. Nuclei were isolated from spleen cells after incubation with LPS by lysis in 0.05% NP 40 (Boehringer Mannheim Biochemicals, Indianapolis, IN) as previously described (32–34). Isolated nuclei were stored at –80° in a glycerol buffer until the run-on reaction was carried out. For the run-on transcription assay, $6–8 \times 10^7$ spleen cell nuclei were incubated with 150 mCi [α-^{32}P]uridine triphosphate for 30 min at 26–28°C. The reaction was stopped by addition of 100 mg tRNA and 100 μg/mL DNase. Proteinase K was added and incubated for 30 min at 42°C. RNA was extracted with phenol-chloroform, passed through G-50 sephadex spun columns (28) (Pharmacia Biotech Inc., Piscataway, NJ), and precipitated with 5% trichloroacetic acid at 0°C. RNA was resuspended and treated with 0.2 M NaOH for 15 min at 0°C. After neutralization, the RNA was precipitated with ethanol and resuspended in the hybridization mix. Denaturation was achieved by heating at

85–90°C for 10 min. Hybridization took place on nitrocellulose filters with each dot containing 5 mg plasmid DNA (35,36). Within each experiment, the same number of counts of nascent RNA, 1–1.6 x 10^7 cpm, was added to each dot.

Fatty acid analysis of spleen cell phospholipids. Spleen cell lipids were extracted with chloroform/methanol (2:1) using a modified Folch method (35). Total phospholipids were isolated by thin-layer chromatography on silica gel G with a solvent consisting of petroleum ether/ether/acetic acid, 70:30:1 (36). The phospholipid band that remained at the origin was scraped from the plate, and phospholipid fatty acids were transmethylated using BF_3. Fatty acid methyl esters were analyzed by capillary gas chromatography as described by Ackman, with minor modifications (37).

RESULTS

Analysis of LPS- and PMA-stimulated IL-1β mRNA. Spleen cells were obtained from mice fed with either BT or FO diets

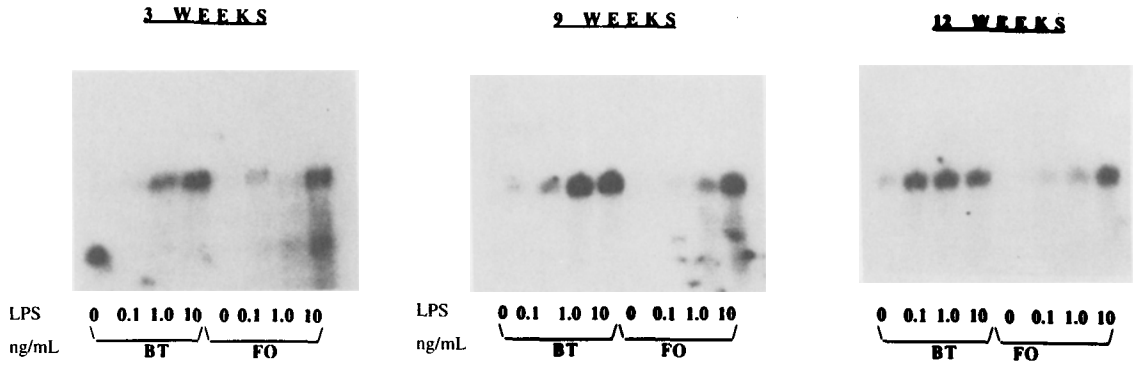


FIG. 1. Northern analysis of total RNA extracts from spleen cells isolated from mice fed for varying time intervals with diets containing either beef tallow (BT) or fish oil (FO) as their lipid components. Cells were stimulated with lipopolysaccharide (LPS) for 4 h prior to RNA extraction. RNA was transferred to nitrocellulose membranes and hybridized with a cDNA probe for murine proIL-1β. Equal quantities of RNA (10 μg) were applied to each lane. Arrows indicate the location of 18S and 28S RNA.

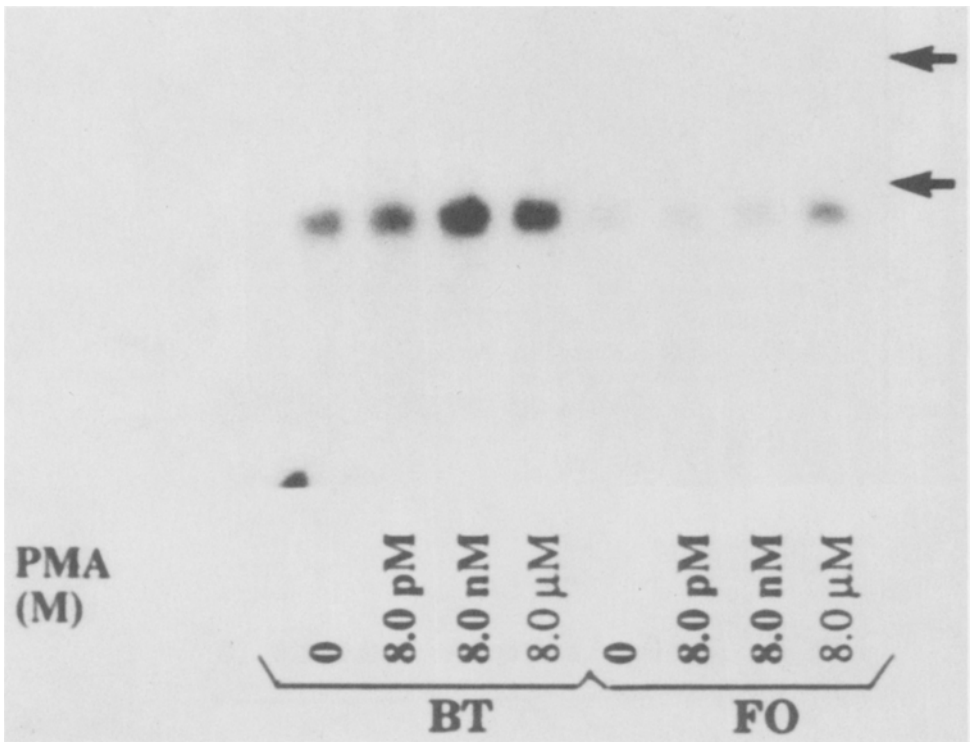


FIG. 2. Northern analysis of proIL-1β mRNA from phorbol 12-myristate 13-acetate (PMA)-stimulated murine spleen cells. Cells were obtained after administration of experimental diets for 12 wk. Abbreviations as in Figure 1.

for varying time periods and were stimulated *ex vivo* to produce IL-1β by either LPS or PMA. In the first experiment (Fig. 1), groups of mice were fed with experimental diets for either 3, 9, or 12 wk prior to stimulation of cells with LPS. In the next experiment (Figs. 2 and 3), mice were fed for 12 wk prior to stimulation of the cells with either LPS or PMA. In each experiment, the amount of LPS-inducible 1.7 kB proIL-1β mRNA derived from FO-fed mice cells was less than that of the BT controls, at most concentrations of LPS or PMA used. In the first experiment (Fig. 1), after 12 wk of feeding, a maximal intensity of the IL-1β mRNA band occurs at 0.1 ng/mL LPS with BT cells. In contrast, only faint bands occur at the lower two concentrations of LPS with FO cells, but at the highest concentration of LPS of 10 ng/mL, intensity of the band is similar to bands with stimulated cells from the BT group. These results are consistent with a lower sensitivity of FO cells than BT cells to LPS, but levels of IL-1β mRNA in most cases are similar in both groups of cells at the highest concentration of LPS.

Results in Figure 2 demonstrate that after PMA stimulation the maximum response of cells from the BT group occurs at 8 nM PMA, whereas in cells from the FO group only the highest concentration of PMA of 8 micromolar produces a signal above background level, and the response observed is less than that seen with the BT group. Aliquots of cells from this experiment were also stimulated with LPS (Fig. 3), with similar results to those seen at 12 wk in Figure 1. This membrane was also hybridized with a human β-actin probe as an internal control for cell numbers and the state of cell differen-

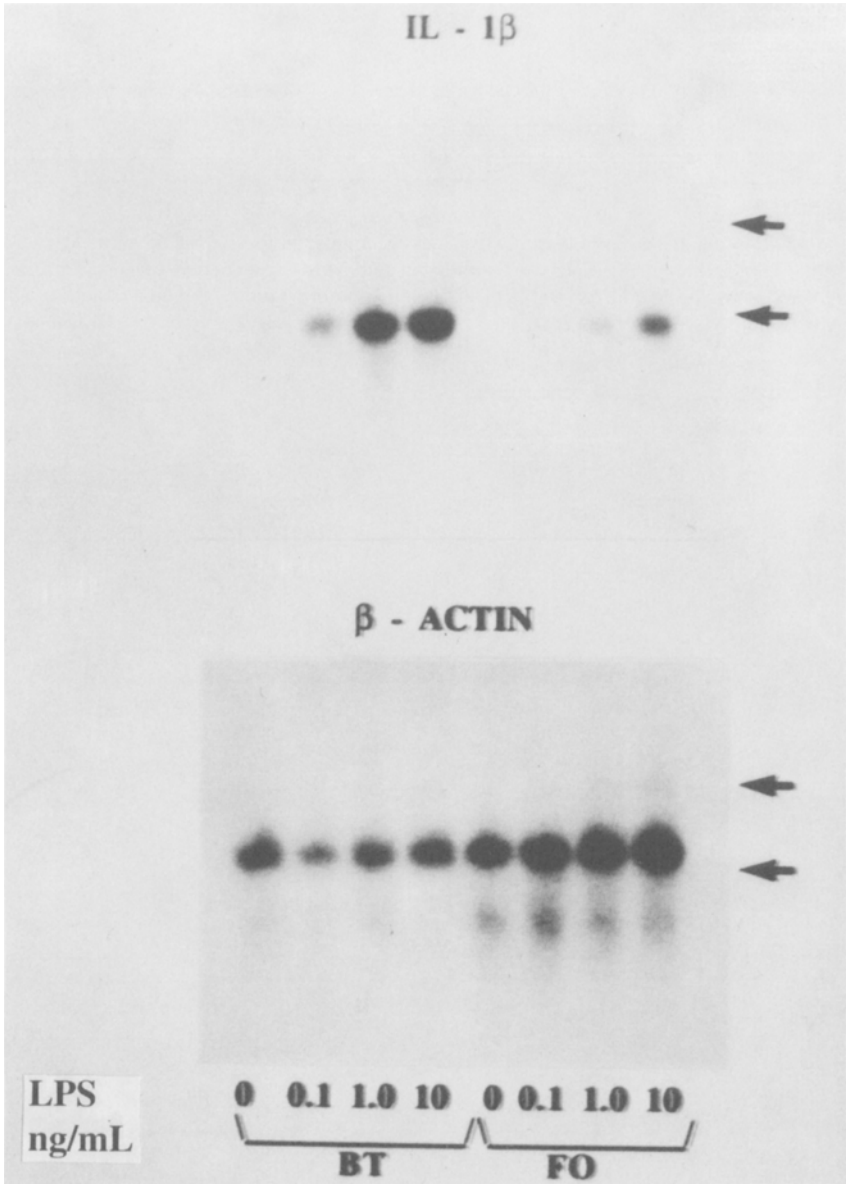


FIG. 3. Analysis of RNA extracts from LPS-stimulated murine spleen cells for proIL-1β and β-actin mRNA. Cells were aliquots of the same preparation used for PMA stimulation in Figure 2. Abbreviations as in Figures 1 and 2.

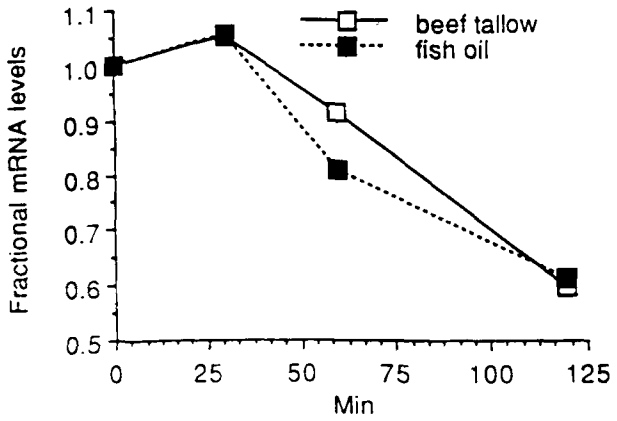


FIG. 4. Degradation rates of IL-1β mRNA from LPS-stimulated murine spleen cells. Cells were stimulated with LPS at 10 ng/mL for 5 h, after which actinomycin D was added to replicate cultures. After incubating with actinomycin D (5 mg/mL) for time periods indicated, each culture was subjected to northern analysis. Quantities of IL-1β mRNA were determined by scanning densitometry. Quantities of mRNA are expressed as the ratios of the densities at each time interval to the densities at the time of addition of actinomycin D (t = 0). Abbreviation as in Figure 1.

tiation. In contrast to results with the IL-1β probe, the β-actin band intensities with LPS stimulation are slightly more intense with the FO group than with the BT group, demonstrating the specificity of IL-1β mRNA effects.

Stability of LPS-stimulated IL-1β mRNA. Since it has been demonstrated that cytokine mRNA have relatively rapid turnover rates, we considered the possibility that reduced levels of IL-1β mRNA generated by cells from FO-treated mice could be achieved by an enhanced rate of decay of the message, compared to the BT group (39). To investigate this possibility, we determined rates of decay of IL-1 mRNA from LPS-stimulated spleen cells after exposure to actinomycin D. Cells were stimulated with 10 ng/mL LPS for 5 h, after which 5 mg/mL actinomycin D were added. Total RNA was isolated from these cells after incubation with actinomycin D for periods up to 2 h and subjected to northern analysis. Results in Figure 4 demonstrate that there is no significant difference in the rates of decay of IL-1β mRNA between the BT and the FO groups (t 1/2 = 2–3 h), indicating that the rate of transcription of the IL-1β gene in these LPS-stimulated cells is reduced in the FO group compared to the BT group.

Nuclear run-on transcription assays. Actinomycin D results suggest that transcriptional regulation is likely to be responsible for the steady-state differences between BT- and FO-fed animals' *ex vivo* cellular responses. Nuclear gene transcription was directly examined by nuclear run-on assay in which isolated nuclei are allowed to complete arrested transcription in the presence of radiolabelled ribonucleotide triphosphates, thus creating probes that can be assessed by hybridization to DNA targets fixed to nitrocellulose membranes. We demonstrated in three separate experiments reduced transcription from the IL-1β gene in LPS-stimulated spleen cells from mice fed the FO diet, compared to mice fed the BT diet. Results of two run-on experiments done in duplicate are shown in Figure 5. In both experiments, no signifi-

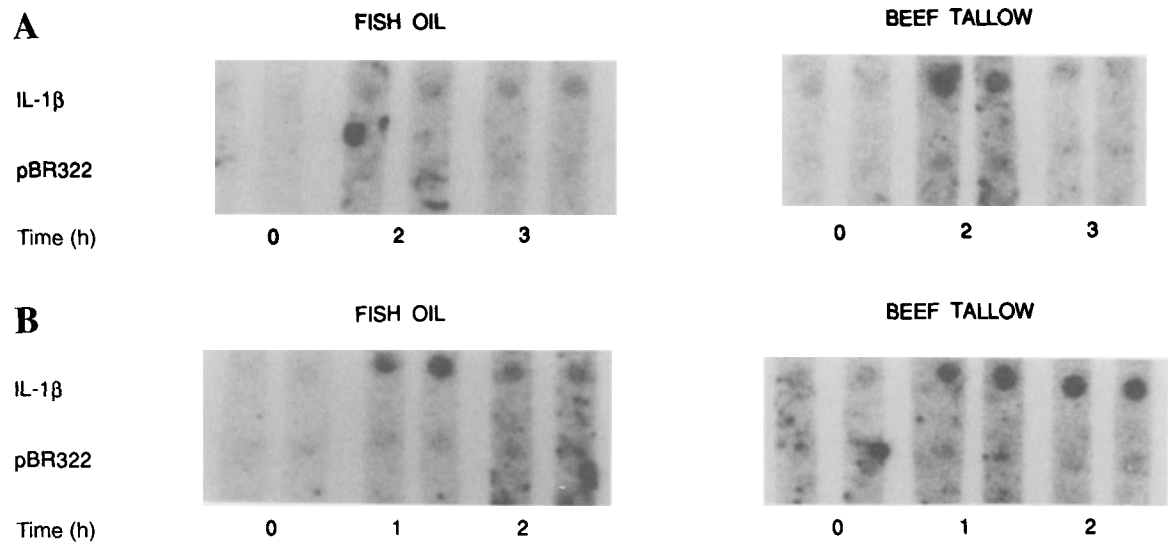


FIG. 5. Nuclear run-on transcripts after LPS stimulation. Nuclei were isolated from murine spleen cells that had been stimulated with LPS (1 ng/mL) for 0 to 3 h as indicated. Radiolabelled nascent RNA transcripts were prepared by incubating 6–8 x 10^7 nuclei with 150 mCi [a-^{32}P]uridine triphosphate. Radiolabelled RNA was isolated from the nuclei by digestion with DNase and protease K followed by phenol-chloroform extraction, G-50 sephadex spun column (Boehringer, Indianapolis, IN) purification, and trichloroacetic acid precipitation. RNA was treated with 0.2 M NaOH, and precipitated with Na acetate-ethanol. Equal counts of RNA (from 1.0 to 1.6 x 10^7 cpm per sample) were used to probe dots on nitrocellulose strips containing 5 mg denatured IL-1β cDNA, or denatured pBR322 plasmid DNA as a control: A) Spleen cells stimulated for 0, 2, and 3 h; B) Spleen cells from different mice than in experiment 1, stimulated for 0, 1, and 2 h. In both experiments, mice were fed described experimental diets for 12 wk before sacrificing to obtain spleen cells.

cant transcription was detected at 0 time, and faint signals were seen after 2 and 3 h of LPS stimulation from FO-fed mice. BT-fed mice showed more activity after 2 h of stimulation than FO-fed mice, with less activity after 3 h. No significant activity was seen with the vector control pBR322 as the target. Figure 5B reveals that there was increased transcription at 1 h of stimulation with RNA from both groups of mice, but at 2 h of stimulation, intense uptake continued with cells from BT mice, while FO cells returned to baseline. Thus, between 0 and 1 h following LPS exposure, the initial rate of IL-1β gene transcription was similar in cells from FO-fed and BT-fed mice. Beyond 1 h, however, transcription in cells from FO-fed mice shut down more rapidly than transcription from BT-fed mice. These findings indicate that dietary n-3 fatty acids reduce the levels of IL-1β mRNA by increasing the efficiency of the rapid gene shutdown associated with the transient expression usually observed for the IL-1β gene (38,50).

Fatty acid analyses of spleen cell phospholipids. Fatty acid analyses of spleen cell preparations were carried out after both 6 and 12 w feeding with each of the experimental diets. The wt% of each polyunsaturated fatty acid present in significant quantities is listed in Table 1. Administration of the FO diet was associated with large increases in the content of long-chain n-3 fatty acid in spleen cell phospholipids. Total polyunsaturated fatty acid content remained nearly constant, regardless of dietary lipid ingested or duration of experimental diets. Elevation of n-3 fatty acid content is therefore accompanied by reduction in n-6 fatty acid content, with relatively small differences in the contents of saturated and monoene fatty acids. The major n-6 fatty acid present is arachidonic acid, which is reduced in cells from the FO group to approximately one-third of the content of arachidonic acid in cells from the BT group. There are also large increases in contents of the three major n-3 fatty acids in cells from the FO group

TABLE 1
Fatty Acid Composition of Total Phospholipids Mouse Speen Cells[a]

	Six weeks[b]		Twelve weeks[b]	
Fatty acid	BT	FO	BT	FO
16.0	21.6	26.8	20.5	26.0
16:1	2.1	4.2	1.4	4.2
18:0	16.8	15.1	17.8	15.7
18:1	18.0	11.4	18.0	11.6
18:2n-6	4.1	1.2	4.1	1.2
20:4n-6	22.0	7.2	24.9	7.9
22:4n-6	2.7	0.2	3.6	0.2
22:5n-6	0.7	0.2	1.5	0.2
20:5n-3	<0.2	11.5	<0.2	11.3
22:5n-3	0.9	6.3	0.7	6.6
22:6n-3	7.3	10.5	4.7	10.3
Saturated	38.8	42.9	38.5	43.2
Monoene	20.9	16.4	20.2	16.6
n-6 PUFA	31.6	11.7	35.9	10.4
n-3 PUFA	8.4	28.8	5.6	28.9
Total PUFA	40.0	40.5	41.5	39.2

[a]Composition determined by weight percentage of fatty acids as their fatty acid methyl ester derivatives; PUFA, polyunsaturated fatty acids; BT, beef tallow; FO, fish oil.
[b]Duration of experimental diets.

DISCUSSION

Results presented in this report demonstrate that dietary n-3 fatty acids inhibit sustained transcription of the IL-1β gene in stimulated spleen cells *ex vivo* by affecting the kinetics of gene expression beyond 1 h poststimulation. Others reported previously that dietary supplements of n-3 fatty acids inhibit production of IL-1 and TNF-α by LPS-stimulated human peripheral blood mononuclear cells (5,6). Studies reported here help clarify the mechanism of cytokine inhibition by n-3 fatty acids. Our experiments demonstrate that dietary n-3 fatty acids reduce steady-state levels of IL-1β mRNA in both LPS- and PMA-stimulated murine spleen mononuclear cells *ex vivo*. Reduced levels of IL-1β mRNA in stimulated spleen cells appear to arise from suppression of IL-1β gene transcription by n-3 fatty acids, based on the reduced rate of transcription beyond 1 h of *ex vivo* cell stimulation as assessed by nuclear run-on assays. Also, similar decay rates for IL-1β mRNA in cells from either FO- or BT-fed mice support the argument for a transcriptional effect. Reduced nuclear transcription does not appear to be related to a decreased induction, but rather to a more rapid shutdown of transcription following induction, as exhibited by the altered kinetics beyond 1 h of treatment. The IL-1β gene was previously shown to be transiently transcribed in various cell lines as well as in peripheral blood monocytes (38). This transient expression is due to the immediate and rapid induction that occurs in the absence of protein synthesis, followed by an almost immediate shutdown resulting from the action of a *de novo* expressed protein, which has been referred to as the transcriptional clamp (23). These data support a model in which either n-3 fatty acids induce a more vigorous expression of the clamp protein(s) or in which BT inhibits clamp expression (compared to alternative fatty acids). Therefore, the role of FO may be to support the strong expression of a protein factor that limits IL-1β gene transcription.

Neither the present nor the previous studies utilized a system containing a single cell type, raising the possibility that the FO effect could be exerting its effect by altering composition of cell preparations from which the cytokines were derived. In the studies reported here, we cannot determine which cell type is responsible for the major portion of IL-1β mRNA, although spleen monocytes are a likely source. We considered the possibility that dietary n-3 fatty acids altered composition of spleen cell preparations such that the percentage of cells containing major quantities of IL-1β mRNA after stimulation was reduced, but the following considerations make this possibility unlikely. No difference in numbers of B-cells and T-cells between BT and FO groups were observed

by fluorescence-activated cell sorter analysis, and no differences in monocytes were observed microscopically. Others also reported that percentages of lymphocytes and monocytes in similar murine spleen cell preparations were not altered significantly by changes in dietary lipids, including n-3 fatty acids (25). The PMA experiment demonstrates that the decreased responsiveness of FO cells is not specific for LPS induction, since the data in Figure 1 demonstrate that responsiveness of FO cells to PMA is less than that of BT cells by over three orders of magnitude.

The mechanism of inhibitory effects of n-3 fatty acids on production of IL-1β mRNA by activated mononuclear cells is unknown. The experimental diets achieved major modifications in the polyunsaturated fatty acid composition of spleen cells, with large reductions in n-6 fatty acids and reciprocal elevations in the contents of n-3 fatty acids, but we have not determined the relationship, if any, between changes in phospholipid fatty acid composition and suppression of IL-1β gene transcription. Our experiments demonstrate that this suppression applies to both a transmembrane and a soluble stimulant, each of which acts by complex, but different, mechanisms (39).

The following evidence indicates that induction of transcription of both IL-1 and TNF-α genes by LPS and PMA may be mediated by activation of protein kinase C. Induction of LPS responsiveness in U937 cells is associated with the stimulation of mRNA synthesis for the G protein Gi2 (40). It was suggested that PMA induces protein kinase C–mediated phosphorylation of endogenous substrates such as the transcriptional activator, AP-1, that could in turn induce transcription of the Gi2 gene. It was also reported that PMA induction of IL-1 by the human monocytic cell line THP-1 was inhibited by a selective protein kinase C inhibitor (41). Since protein kinase C utilizes both diacylglycerol and phospholipids as cofactors, it is possible that the changes in fatty acid composition of phospholipids associated with higher levels of dietary n-3 fatty acids could inhibit the activity of protein kinase C due to changes in the structures of its lipid cofactors.

Certain eicosanoids have been found to modify the production of cytokines. Inhibition of LPS-induced TNF-α mRNA production from murine peritoneal macrophages was observed with prostaglandin E_2 ($PGE_{1)}$ and PGE_2, but leukotriene B_4 (LTB_4), and LTC_4 had no effect (42). Conversely LTB_4 and LTD_4 enhanced production of IL-1 by human peripheral blood monocytes that had been stimulated by LPS and other agonists (43). Since dietary marine lipids reduce production of 5-lipoxygenase products by human leukocytes (6,15), it is possible that suppressed LT production by mononuclear cells from FO-treated mice could reduce IL-1 production, perhaps by interfering with a key early signaling requirement. Another study, however, reported that LTB_4 did not enhance LPS-stimulated IL-1 production by murine peritoneal macrophages (44), and thus LT enhancement of IL-1 synthesis may be limited to certain cell types. We have not yet examined effects of LT on IL-1 production by spleen cell preparations utilized in experiments reported here.

Dietary n-3 fatty acids also suppress formation of prostaglandins and thromboxanes, although production of prostacyclin in humans is not affected significantly (45–48). Although the E prostaglandins suppress TNF-α mRNA production, as noted above, and also suppress production of IL-1 protein (44,49), evidence indicates that inhibition of IL-1 production is at a post-transcriptional level, since IL-1β mRNA levels are unaffected in stimulated U937 cells by PGE_2 (49). In any event, the E prostaglandins inhibit cytokine production, and thus the usual reduction of PGE levels by n-3 fatty acids, if such inhibitory effects occurred in our experimental system, would have augmented cytokine mRNA levels.

This study demonstrates that dietary n-3 fatty acids suppress levels of IL-1 mRNA in murine spleen cells following activation by two different stimuli, and results with LPS-stimulated cells indicate that the mechanism involves inhibition of induction of IL-1 gene transcription by n-3 fatty acids. Molecular mechanisms for inhibition of IL-1 gene transcription by n-3 fatty acids remain to be determined. IL-1 gene expression depends on the integrity of an upstream LPS and PMA responsive enhancer and a tissue-specific promoter, and both of these regions bind positive factors important for gene expression. This raises the possibility that n-3 fatty acids may interfere with the function of factors necessary for maximal IL-1 gene induction.

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Polyunsaturated Fatty Acids Inhibit Hepatic Stearoyl-CoA Desaturase-1 Gene in Diabetic Mice

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ABSTRACT: Insulin and dietary fructose independently induce stearoyl-CoA desaturase 1 (SCD1) gene expression in diabetic mouse liver. In the present study, we again used diabetic mice and supplemented a high fructose diet with polyunsaturated fatty acids (PUFA) to determine the selective repression of SCD1 gene expression by dietary PUFA, as previously shown in normal mice. We saw dramatic repression of SCD1 mRNA expression, with trilinolenin at 3% and triarachidonin at 1% supplementation. We also observed significant repression of insulin-induced SCD1 mRNA upon supplementation of the noninducing starch diet with PUFA. In conclusion, our data demonstrate that PUFA negatively regulate hepatic gene expression through an insulin-independent mechanism.

Lipids 31, S-33–S-36 (1996).

Stearoyl-CoA desaturase (SCD) is a key enzyme involved in biosynthesis of unsaturated fatty acids and regulation of this process. The enzyme catalyzes the Δ^9-*cis* desaturation of C_{16} and C_{18} fatty acyl-CoAs, leading to synthesis of palmitoleoyl- and oleoyl-CoA as major products (1). Hepatic SCD enzyme levels, like a number of lipogenic enzymes, are increased by a high-carbohydrate diet (2), and this increase in enzyme activity correlates positively with the abundance of SCD mRNA in liver (3,4). We showed that this increase in stearoyl-CoA desaturase gene 1 (SCD1) mRNA is due to enhancement in the transcriptional activity of the SCD1 gene (5). Recently, we observed that the increase in SCD1 mRNA caused by fructose and insulin can occur independently in diabetic mice, whereas the transcriptional induction requires both inducers (6). This is consistent with other lipogenic genes, such as fatty acid synthase and S14, which are known to be transcriptionally activated by insulin and fructose (7,8), leading to stimulation of lipogenesis in liver.

Polyunsaturated fatty acids (PUFA) have been shown to reduce genetic expression of many enzymes in the biosynthesis of fat, including SCD1, fatty acid synthase, acetyl-CoA carboxylase, and malic enzyme (5,9–11). A fat-free, high-carbohydrate diet supplemented with 3% triarachidonin (20:4) represses induced SCD1 mRNA by more than 95% and transcription by 75% (5). Arachidonic and eicosapentaenoic (20:5) acids have also been shown to reduce SCD1 mRNA levels in cultured primary hepatocytes by as much as 90% (12). This dramatic repression suggests that PUFA, or some PUFA metabolite, have a distinct and significant role in lipogenic gene regulation.

In this study, we examined the mechanism of PUFA repression of SCD1 mRNA in diabetic mice to determine if this repression was insulin dependent. We found that both trilinolenin (18:3) and triarachidonin significantly repress fructose-induced SCD1 mRNA in insulin-deficient mice, as shown by RNase protection analysis. Both trilinolenin and triarachidonin also repress insulin-induced SCD1 mRNA when diabetic mice are given a physiological dose of regular and lente (slow-acting) insulin. None of these manipulations affect β-actin mRNA levels, showing that this effect is specific to SCD1. Overall, our results show that PUFA repress SCD1 mRNA through an insulin-independent mechanism.

MATERIALS AND METHODS

Fructose and starch diets were made in our laboratory using appropriate sugars obtained from Fisher Scientific (Springfield, NJ) and Purina Formulab Diet 5008 (Richmond, IN). Regular (bovine) insulin was purchased from Sigma (St. Louis) and lente (porcine) insulin was from Eli Lilly Corp. (Indianapolis). cDNA for the β-actin riboprobe was purchased from Ambion, Inc. (Austin). All plasmid DNA were isolated by modification of the SDS-NaOH method (13), and nucleic-acid–modifying enzymes were purchased from Promega Biotechnology Inc. (Madison), Boehringer Mannheim (Indianapolis) and New England Biolabs (Beverly, MA). Butylated hydroxytoluene, streptozotocin, trilinolenin, and triarachidonin were all obtained from Sigma.

Animals and treatments. Male BALB/c mice, used throughout this study, were obtained from the Department of Biochemistry of the University of Wisconsin in Madison. Mice (4–6 weeks old) were made diabetic by four daily intraperitoneal

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Abbreviations: SCD1, stearoyl-CoA desaturase gene 1; SCD2, stearoyl-CoA desaturase gene 2; PUFA, polyunsaturated fatty acids; PPAR, peroxisome proliferator-activated receptors.

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injections of streptozotocin (5 mg/100 g) in sodium citrate (pH 4.5), as described previously (6). Diabetes was confirmed by high fasting glucose levels determined by using Clinetex (Fisher Scientific) and animals were considered diabetic when blood glucose levels exceeded 250 mg/dL. Animals were maintained on a complete diet of Purina Formulab Diet 5008 throughout the entire course of streptozotocin injections. Once diabetic, mice were fasted for 12 h and then switched from the chow diet to either an 80%-fructose or 80%-starch diet for 24 h. When stated, trilinolenin and triarachidonin were supplemented at 3 and 1%, respectively, with 0.1% butylated hydroxytoluene as an antioxidant, and all diets were made fresh daily. Insulin was administered to diabetic animals at a combined dose of regular insulin (3 units/100 g) intraperitoneally and lente insulin (30 units/100 g) subcutenously. All animals were given free access to tap water. Blood glucose levels were again measured upon sacrifice for all mice to check for proper hormonal status.

Measurement of mRNA. Total hepatic RNA was isolated according to Chirgwin et al. (14), and RNase protection analysis of SCD1 and β-actin mRNA expression was performed as described previously (6). Quantification of relative mRNA was determined by laser densitometric scanning (Molecular Dynamics, Sunnyvale, CA) of autoradiograms. Integrity of the RNA used for the RNase protection analysis was verified by fractionating 30 μg total liver RNA on a 1% agarose gel containing 6.7% formaldehyde and visualizing RNA by ethidium bromide staining.

RESULTS

Effect of PUFA on fructose induction of SCD1 mRNA in diabetic mice. SCD1 is transcriptionally induced upon fasting and then refeeding a fat-free, high-carbohydrate diet. Dietary PUFA have been shown to repress transcription of SCD1 in livers of normal mice (5). We have recently shown that fructose and insulin are capable of inducing SCD1 mRNA independently; however, transcriptional induction requires both fructose and insulin. To determine whether the PUFA-mediated repression of SCD1 was insulin dependent, diabetic mice were fasted and refed an 80%-fructose diet with or without supplemented PUFA. Trilinolenin was supplemented at 3%, and triarachidonin was supplemented at 1%; SCD1 mRNA levels were analyzed by RNase protection analysis using SCD1- and β-actin complementary riboprobes (Fig. 1A). Autoradiograms were subjected to laser densitometric scanning, and results of this are shown in Fig. 1B. Trilinolenin repressed fructose-induced SCD1 mRNA levels by 90% and triarachidonin by 93%, whereas β-actin mRNA levels were not significantly changed. These results show that PUFA regulate SCD1 gene expression via an insulin-independent mechanism. This is consistent with Wilson et al. (15), who observed that dietary unsaturated fat could repress overall liver lipogenesis in severely diabetic rats, whereas saturated fat had no effect.

Effect of PUFA on insulin induction of SCD1 mRNA in diabetic mice. Figure 1 showed that PUFA regulate SCD1 in insulin-deficient mice. To determine if PUFA can repress insulin induction of SCD1 mRNA in the absence of inducing carbohydrate, diabetic mice were fasted and then refed a noninducing 80%-starch diet for 24 h. Insulin was administered for 18 h before sacrifice. Where indicated, trilinolenin and triarachidonin were supplemented to the starch diet at 3 and 1%, respectively. SCD1 and β-actin mRNA levels were measured by RNase protection and quantitated as described above.

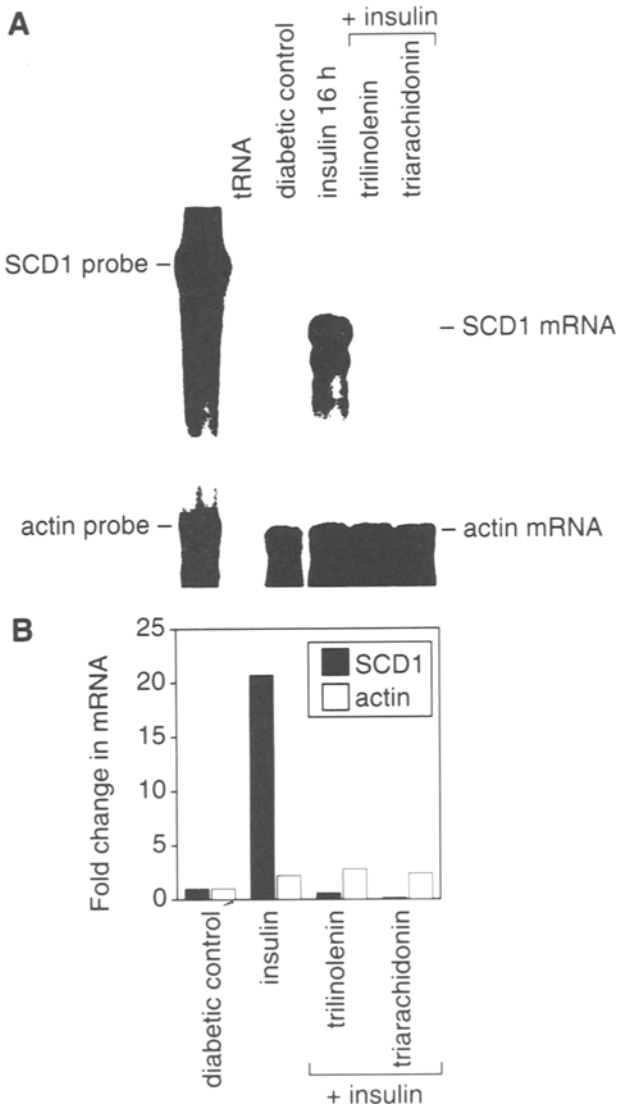


FIG. 1. Repression of fructose-induced SCD1 mRNA by trilinolenin and triarachidonin. Mice were made diabetic by 4 daily injections of streptozotocin and were considered diabetic when blood glucose levels reached > 250 mg/dL. Mice were then fasted for 12 h and switched from chow diet to 80%-fructose diet for 24 h. Where indicated, trilinolenin and triarachidonin were supplemented at 3 and 1%, respectively. Livers were harvested, and total RNA was isolated as described. (A) SCD1 and β-actin mRNA were measured by RNase protection analysis using radiolabeled complementary riboprobes and visualized by autoradiography. (B) autoradiograms in A were subjected to laser densitometry to measure relative change in mRNA, and data are normalized to control diabetic animal. Films of differing exposures were scanned, giving essentially identical results.

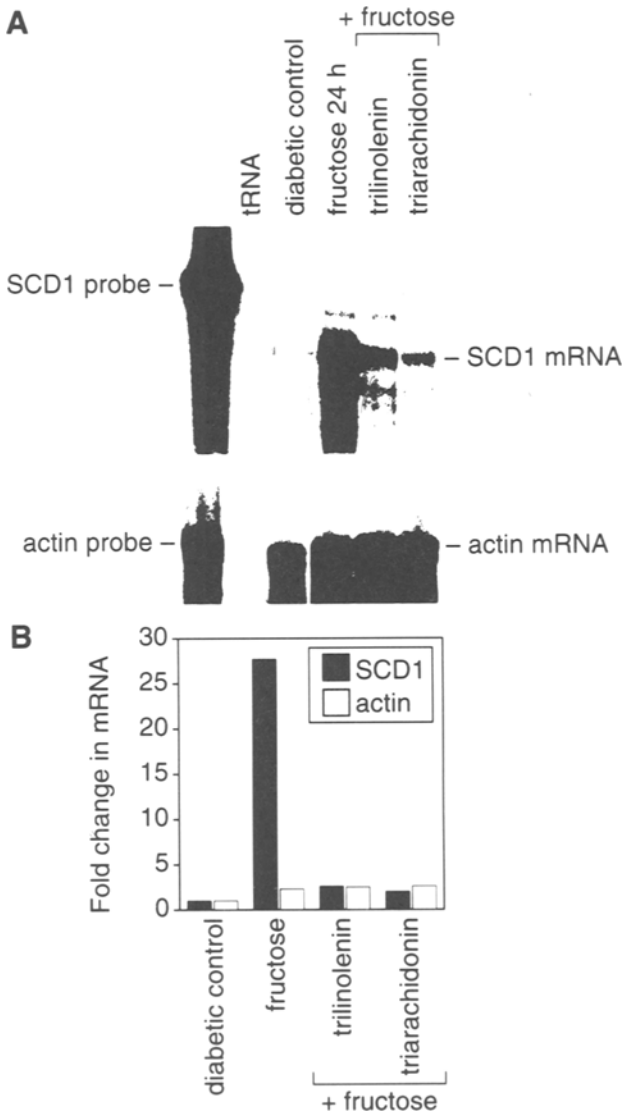


FIG. 2. Repression of insulin-induced SCD1 mRNA by trilinolenin and triarachidonin. Mice were made diabetic as described. Once diabetic, mice were fasted for 12 h and then switched from chow diet to 80%-starch diet for 24 h, with trilinolenin and triarachidonin supplemented at 3 and 1%, respectively, where stated. Insulin was given in a combined dose of 3 U/100 g regular insulin intraperitoneally and 30 U/100 g lente insulin subcutaneously. Livers were harvested, and total RNA was isolated as described. (A) SCD1 and β-actin mRNA were measured as for fructose, and autoradiograms are shown; (B) autoradiograms in A were subjected to laser densitometry, and data are normalized to control diabetic animals.

Autoradiograms and data are shown in Fig. 2. As with fructose, trilinolenin and triarachidonin repressed SCD1 mRNA levels by 97 and 99%, respectively, and again β-actin mRNA levels were not significantly altered. Control animals receiving insulin vehicle injections showed no effect on SCD1 or β-actin mRNA levels (data not shown). This study shows that PUFA repress insulin-dependent SCD1 mRNA levels in the absence of inducing carbohydrate and that these manipulations are specific to SCD1.

DISCUSSION

Stearoyl-CoA desaturase genes (SCD1 and SCD2) encode a key enzyme involved in biosynthesis of unsaturated fatty acids and in regulation of this process. Hepatic SCD1 transcription has been shown to increase by fasting and then refeeding a fat-free, high-carbohydrate diet to animals (5); it decreases upon supplementation of the fat-free diet (or media) with PUFA (5,12). In a recent study, we found that the full induction of SCD1 mRNA observed previously in the normal mouse could not be restored by either insulin or fructose alone, but instead required a combination of the two or a combination of glucose and insulin (6). In this study, we have shown that PUFA repress SCD1 mRNA in diabetic mice independently of insulin. Trilinolenin and triarachidonin suppress fructose-induced SCD1 mRNA levels by 90 and 93%, respectively, with no effect on β-actin levels. Likewise, trilinolenin and triarachidonin repress insulin-induced SCD1 mRNA levels by 97 and 99%, respectively, again with no effect on β-actin levels. We believe that PUFA are repressing SCD1 transcription, as well as posttranscriptional regulation of mRNA, since transcriptional repression of SCD1 by PUFA has been shown previously in normal animals (5).

Repression of fatty acid biosynthesis by PUFA is not universally found in all tissues. Rat adipose tissue lipogenesis is repressed by dietary long-chain fat supplementation, but to a lesser extent than in liver tissue (16), and rat lung tissue lipogenesis has been shown to be resistant to repression by dietary unsaturated fat (17). Medium-chain-length triglyceride has been shown to induce lipogenesis in liver of diabetic rats in an insulin-dependent manner (18), apparently by a distinct and separate mechanism than what we are observing with PUFA. Dietary polyunsaturated fat has also been shown to reduce triiodothyronine's induction of rat liver fatty acid synthase and malic enzymes in a dose-dependent manner (19).

For stearoyl-CoA desaturase, the picture still remains unclear. Cultured lymphocytes exhibit similar repression of SCD2 transcription by arachidonic acid as SCD1 in hepatocytes (20), whereas linoleic acid is suggested to induce SCD2 mRNA in neonatal mouse brain (21). Many studies of PUFA repression of lipogenic genes have been reproduced in primary hepatocyte culture (12,22–23), and a PUFA-responsive element has been localized for fatty acid synthase (24) and S14 (25) genes. It has been suggested that PUFA repression of lipogenic genes is mediated by peroxisome proliferator-activated receptors (12). We do not believe this to be the case, since peroxisome proliferators actually induce SCD1 mRNA in mouse liver [Miller, C.W., and Ntambi, J.M., (1995) submitted for publication], unlike the repression seen with PUFA. This suggests that SCD1 may be regulated by some mechanism unique from that of other lipogenic genes. Details of this mechanism remain unknown at the time of this report.

Another recent study showed that SCD1 mRNA is repressed by arachidonic acid in cultured H2.35 liver cells (26), and we have found that this repression can be transcriptionally reproduced using chloramphenicolacetyltransferase reporter gene constructs containing portions of the SCD1 promoter region

(Sessler, A.M., and Ntambi, J.M., unpublished data). This immortal cell culture model provides a tool for identifying SCD *cis*-acting elements without the complications of primary hepatocyte culture or transgenic animal studies. It is hoped that more precise identification of the carbohydrate/insulin-, PUFA-, and peroxisome proliferator-dependent factors that regulate SCD1 gene expression will be achieved with these H2.35 liver cells in culture.

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Effects of Different Polyunsaturated Fatty Acids on Growth-Related Early Gene Expression and Cell Growth

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ABSTRACT: Effects of polyunsaturated fatty acids on expression of early-response genes c-*fos* and Egr-1 and induction of cell growth were assessed in Swiss 3T3 fibroblasts. Stimulation with arachidonic acid increased mRNA levels of c-*fos* and Egr-1. This effect was inhibited by preincubation with cyclooxygenase inhibitors and restored by addition of prostaglandin E_2 (PGE_2), the predominant eicosanoid produced in Swiss 3T3 fibroblasts. Further signaling of PGE_2 was mediated by a protein kinase C–dependent pathway, since downregulation, or inhibition, of protein kinase C reduced increases in mRNA levels. Parallel to the stimulatory effects on mRNA levels, AA and PGE_2 also increased cell growth, as determined by uptake of [^{3}H]-thymidine. In contrast to arachidonic acid, n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) did not increase c-*fos* and Egr-1 mRNA levels or cell growth. Furthermore, preliminary data indicate that EPA and DHA even reduce the stimulatory effect of AA, which is associated with reduced formation of PGE_2. In conclusion, our data indicate that AA increases expression of growth-related early genes c-*fos* and Egr-1 in Swiss 3T3 fibroblasts by its conversion to PGE_2 and subsequent activation of protein kinase C, whereas n-3 fatty acids do not activate this signaling cascade.
Lipids 31, S-37–S-40 (1996).

Arachidonic acid (AA) and its metabolites have recently been identified as a novel group of intracellular second messengers (1) regulating ion channels and activities of enzymes involved in signal transduction, such as GTPase-activating protein or protein kinases (1,2). In addition, AA and its metabolites have been found to modulate gene expression. Endogenous formation of cyclooxygenase or noncyclooxygenase metabolites of AA has been implicated to mediate expression of early-response genes c-*fos*, Egr-1, c-*myc*, or c-*jun* in different cell types after mitogenic stimulation (3,4) or treatment with H_2O_2 (5,6). Furthermore, stimulation with lipoxygenase or cyclooxygenase metabolites of AA also increased early gene expression (7–9), indicating that endogenously produced eicosanoids may modulate early gene expression in an autocrine or paracrine way. In addition, a more direct effect of polyunsaturated fatty acids (PUFA) on the expression of gene coding for enzymes of fatty acid metabolism has been reported. PUFA, including AA and other n-6 and n-3 fatty acids, transcriptionally repressed expression of the fatty acid synthase gene (10), the hepatic S_{14} gene (11), and the genes coding for stearoyl-CoA desaturase (12). Based on chimeric gene constructs (10,11), an AA response element has been identified but not yet sequenced.

Apart from modulation of gene expression, AA and n-3 PUFA have also been implicated to differentially influence cell growth. Most observations indicate a promitogenic activity of n-6 PUFA (4,13,14), whereas n-3 PUFA have been reported to mainly reduce pathologic growth (15–17). We studied Swiss 3T3 fibroblasts to further delineate signaling pathways involved in the regulation of early gene expression and possibly cell growth by PUFA. We characterized a novel mechanism by which AA induces c-*fos* and Egr-1 expression (18) and found evidence that n-3 PUFA differentially influence gene expression and cell growth in 3T3 fibroblasts.

EXPERIMENTAL PROCEDURES

Tissue culture. Tissue culture conditions for Swiss 3T3 fibroblasts were essentially as described (18). In particular, cells were regularly split every seven days, and all experiments were performed with confluent cultures.

Northern blot analyses. After stimulation for 30 min, RNA extraction and Northern blot analyses were performed essentially as described previously (18). For hybridization, cDNA coding for the 0.48 kb human c-*fos* fragment from exon 4, the 2.6 kb mouse Egr-1 fragment, and the 0.8 kb rat GAPDH fragment were subcloned in riboprobe vectors. RNA transcribed from these riboprobe vectors was used as probes and hybridized to the blots, as detailed elsewhere (18). The same blot was hybridized to all three probes in two steps.

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Abbreviations: AA, arachidonic acid; cAMP, cyclic adenosine monophosphate; DHA, docosahexaenoic acid; EGF, epidermal growth factor; EP, ; EPA, eicosapentaenoic acid; HPLC, high-performance liquid chromatography; PG, prostaglandin; PKC, protein kinase C; PMA, phorbol myristate acetate; PUFA, polyunsaturated fatty acids; TCA, trichloroacetic acid.

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Determination of thymidine incorporation. Cell growth was determined by incorporation of [^{3}H]-thymidine. Cells were serum-deprived for 24 h and stimulated by addition of PUFA, prostaglandin E_2 (PGE_2), or epidermal growth factor (EGF) for 24 h in the presence of 0.5 μCi [^{3}H]-thymidine. trichloroacetic acid (TCA)-precipitable radioactivity was quantified by liquid scintillation counting in a β-counter.

Metabolism of [^{14}C]-arachidonic acid. Eicosanoid formation was analyzed as detailed previously (18,19). Cells were stimulated with 10 μM AA [0.5 μCi (1 μM) [^{14}C]-AA plus 9 μM nonradioactive AA] for 15 min. After fluid extraction, eicosanoids were separated by reverse-phase high-performance liquid chromatography (HPLC) (19). Metabolites were identified by their characteristic retention times and by coelution of authentic standards. Eicosanoids were quantified after correction for the recoveries of the internal standard PGB_2 added prior to extraction and of total radioactivity in each sample. In addition, PGE_2 formation in cells stimulated with 10 μM AA, eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) was quantified by ELISA (Cascade Biochemicals, Reading, United Kingdom), as recommended by the manufacturer.

RESULTS

Stimulation with AA (2.5–10 μM) or with the calcium ionophore A23187 (10 μM), which activates phospholipase A_2 to release endogenous AA from phospholipids, induced accumulation of c-*fos* and Egr-1 mRNA. Preincubation with the cyclooxygenase inhibitor indomethacin (0.1–10 μM) or other cyclooxygenase inhibitors markedly reduced induction of early gene expression by AA, indicating that intrinsic cyclooxygenase activity is required to mediate the effect of AA.

To identify the cyclooxygenase metabolite, 3T3 cells were stimulated with [^{14}C]-AA alone, and lipid extracts were analyzed by HPLC. The predominant eicosanoid produced spontaneously was found to be PGE_2, as evidenced by its specific retention time on HPLC and cochromatography of authentic standards. In addition, using monocytic U937 cells, we previously confirmed by gas chromatography–mass spectrometry analysis that a compound eluting with the same retention time on HPLC was PGE_2 (19).

Given that PGE_2 is the main metabolite of AA in 3T3 fibroblasts, its effect on c-*fos* and Egr-1 mRNA levels was determined. PGE_2 (10 μM) alone increased accumulation of both mRNA to the same degree as did AA. Furthermore, PGE_2 reversed the inhibitory effect of indomethacin on mRNA accumulation in 3T3 cells stimulated with AA, indicating that endogenous conversion of AA to PGE_2 mediates induction of early gene expression.

PGE_2 has been found to transduce its signal by binding to at least three different receptor subtypes (EP_{1-3}; 20), which activate different second messenger systems. Binding to the EP_1 receptor stimulates phospholipase C, leading to generation of inositolphosphates and diacylglycerol, which finally result in increased cytosolic free calcium levels and activation of protein kinase C (PKC). In contrast, activation of EP_2 and EP_3 receptor subtypes either enhance (EP_2) or decrease (EP_3) adenylate cyclase activity; resulting in respective changes in cAMP levels.

To further characterize intracellular signaling pathway(s) activated by PGE_2, we determined intracellular cAMP levels and evaluated effects of PKC inhibition and of agents known to increase cAMP on the levels of c-*fos* and Egr-1 mRNA. In cells stimulated with AA or PGE_2, cAMP levels were only 1.5-fold higher, as compared to unstimulated cells, whereas treatment with forskolin (50 μM), a compound that directly activates adenylate cyclase, resulted in a 15-fold increase. Similarly, agents known to increase cAMP formation [forskolin and dibutyryl cAMP (10–100 μM)] did not increase mRNA levels in a manner comparable to AA or PGE_2, indicating that activation of adenylate cyclase is not involved in AA- or PGE_2-induced expression of c-*fos* and Egr-1. In contrast, downregulation of PKC by prolonged stimulation with phorbol myristate acetate (PMA) (800 nM) or inhibition of PKC by chelerythrine (20 μM) or calphostin C (0.5 μM) almost completely inhibited c-*fos* and Egr-1 mRNA accumulation in cells stimulated with AA or PGE_2.

Taken together, these data indicate that exogenously added or endogenously released AA induces c-*fos* and Egr-1 mRNA accumulation by its endogenous conversion to PGE_2 and subsequent activation of PKC, possibly by binding to an EP receptor subtype linked to activation of phospholipase C (Fig. 1).

Increased expression of early genes has been reported to occur as an early response to mitogenic stimulation (21,22). To further relate increases in c-*fos* and Egr-1 mRNA levels to cell growth, the effect of AA and PGE_2 on uptake of [^{3}H]-thymidine was assessed. Both AA (10 μM) and PGE_2 (1 μM) increased thymidine incorporation by 167 ± 32% and 150 ± 21%, respectively.

In contrast to AA or PGE_2, treatment with the n-3 PUFA EPA (10 μM) or DHA (10 μM) increased neither thymidine

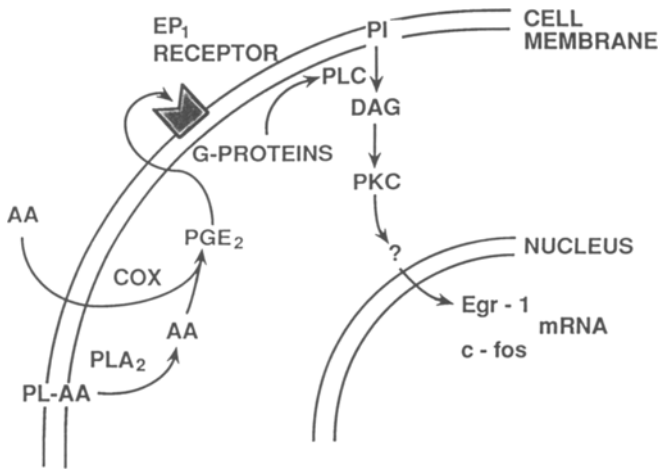


FIG. 1. Proposed signaling cascade for induction of c-*fos* and Egr-1 mRNA expression by arachidonic acid in Swiss 3T3 fibroblasts.

TABLE 1
Effects of AA, EPA, or DHA on Thymidine Incorporation and PGE_2 Formation[a]

PUFA added	AA[b]	EPA[c]	DHA[d]
Thymidine uptake (% control)	167 ± 32	112 ± 20	103 ± 12
PGE_2 formation (ng/mL)	3237 ± 247	147 ± 137	70 ± 12

[a]Data are presented as mean ± SD from three independent experiments. In the control condition, cells were treated with the vehicle for polyunsaturated fatty acids (PUFA), ethanol, only. [b]AA = Arachidonic acid. [c]EPA = Eicosapentaenoic acid. [d]DHA = Docosahexaenoic acid.

incorporation nor formation of PGE_2 (Table 1). Furthermore, preliminary data indicate that EPA and DHA do not induce accumulation of c-*fos* and Egr-1 mRNA and even antagonize effects of AA on gene expression and cell growth. This is paralleled by decreased PGE_2 formation, indicating that antagonistic effects of n-3 and n-6 PUFA on early gene expression and cell growth are mediated by differential stimulation of PGE_2 formation and subsequent activation of PKC.

DISCUSSION

Among the many biochemical activities of AA and its metabolites, regulation of gene expression has recently been identified as a novel property of PUFA and eicosanoids. Apart from genes coding for enzymes of fatty acid metabolism (10,12,23), eicosanoids have been found to modulate the expression of various early-response and growth-related genes (3–6), many of them coding for transcription factors. The molecular mechanism underlying modulation of gene expression by PUFA are not yet fully characterized, however. Studying genes coding for enzymes of fatty acid metabolism, deletion analyses, and chimeric constructs suggested that AA and other n-3 and n-6 PUFA may directly interact with a fatty acid response element (10,11), although its nucleotide sequence has not yet been identified. Furthermore, n-3 and n-6 PUFA have been found to stimulate transcriptional activity of the promoter of the acyl-CoA oxidase gene, which is required for fatty acid ß-oxidation, by activation of the peroxisome proliferator activator receptor (23), a transcription factor belonging to the superfamily of nuclear receptors.

Although these observations indicate a direct transcriptional activity of PUFA, this has not been confirmed by studying early genes. Accordingly, we found that in Swiss 3T3 fibroblasts AA induces expression of c-*fos* and Egr-1 after its conversion to PGE_2 and subsequent activation of PKC. This signaling cascade has essentially been confirmed in a study by Simonson *et al.* (24), demonstrating that, in rat mesangial cells, AA and PGE_2 induce c-*fos* and c-*jun* expression through a cAMP-independent and PKC-dependent mechanism. Both studies indicate that activation of a PGE_2 receptor on the plasma membrane, possibly the EP_1 receptor subtype, is involved to further stimulate PKC and transduce the signal to the nucleus.

In conclusion, our observations and those reported by Simonson *et al.* (24) provide evidence that, in addition to a direct transcriptional effect of fatty acids, AA induces expression of early genes by an autocrine or paracrine mechanism requiring its endogenous conversion to the cyclooxygenase metabolite PGE_2, which further activates PKC-dependent intracellular signaling to increase early-gene mRNA accumulation. Consistent with this intracellular signaling cascade, antagonistic effects of n-3 fatty acids EPA and DHA on AA-induced mRNA accumulation and cell growth can at least in part be explained by their inhibitory effect on PGE_2 formation.

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The Cardiac Antiarrhythmic Effects of Polyunsaturated Fatty Acid

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ABSTRACT: Each year in the United States alone some 250,000 persons die within one hour of an acute myocardial infarction. These deaths are largely due to ischemia-induced ventricular arrhythmias, primarily ventricular fibrillation (VF). Thus a safe, simple means of preventing such arrhythmias has considerable public health benefit potential. We have demonstrated that the intravenous infusion of n-3 polyunsaturated fatty acids (PUFA) from fish oils will prevent ischemia-induced VF in prepared, nonanesthetized, exercising dogs, confirming earlier feeding studies in rats. We show that this protective effect is due to an action of the free acidic form of the PUFA to alter the electrophysiology of individual cardiac myocyte so that the cells are electrically more stable. The electrophysiologic effects, in turn, result from direct and specific effects of the PUFA to block the fast voltage-dependent sodium channels. The binding of the free fatty acids is directly to the protein of the sodium channels and results in prolongation of the inactivated state of these channels. Other ion channels are also affected by the PUFA. Two clinical trials with n-3 PUFA are mentioned which inadvertently support the antiarrhythmic potential of PUFA ingestion.
Lipids 31, S-41–S-44 (1996).

There has been suggestions prior to the 1980s that polyunsaturated fatty acids (PUFA) are antiarrhythmic (1,2). McLennan and Charnock (3–5) have shown this to be the case in studies in rats fed diets high in saturated fat, olive oil, sunflower seed oil or tuna fish oil. Both saturated fat and olive oil resulted in a high incidence of irreversible ventricular fibrillation (VF) when an experimental myocardial infarction was produced. The sunflower seed oil reduced the high incidence of VF, but the tuna fish oil prevented the malignant arrhythmia entirely. With Billman, we have confirmed directly the prevention of cardiac sudden death from VF in an arrhythmia-prone dog model (6). Now in 11 of 14 prepared, conscious, exercising dogs tested, the ischemia-induced VF has been prevented by an intravenous infusion of a fish oil emulsion containing primarily a mixture of eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA) just prior to the ischemic insult ($P < 0.006$). The intravenous route of administration was used after we had found that the action of these long chain polyunsaturated fatty acids occurred quickly and to demonstrate unequivocally that the protective effect is caused by these to n-3 fatty acids and not by some other constituent of the diets or fish oil.

Our laboratory has been studying the mechanism of the antiarrhythmic action of polyunsaturated fatty acids. The neonatal rat cardiac myocyte preparation has proven useful for these studies. The hearts of 1–2 d old rats are treated with trypsin and collagenase to separate individual myocytes which are cultured in appropriate media (7). By the third day the cells have grown into clumps of syncytia which beat spon-

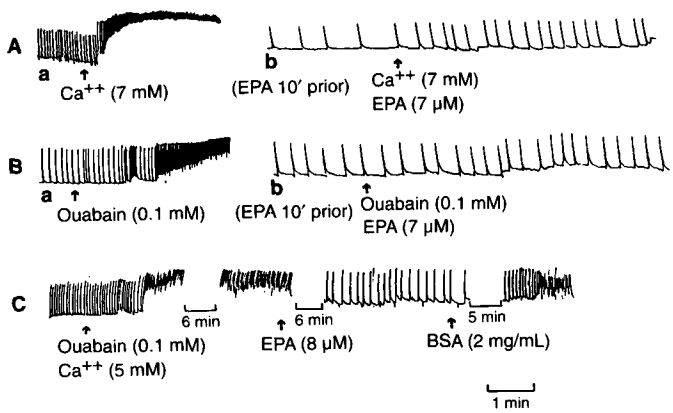


FIG. 1. Tracings show prevention and termination of arrhythmias by (EPA). Perfusion of the myocytes with a solution containing 7 mM calcium (Aa) or 0.1 mM ouabain (Bb) induced contracture and fibrillation of the myocytes. Washing the cells with medium containing 1.1 mM calcium for 5–8 min. returned the rhythm to the control beating rate. Then the cells were perfused with a medium containing 7 mM EPA. After 10 min when the beating rate had slowed, addition of the 7 mM calcium (Ab) or 0. I mM ouabain (Bb) failed to induce contracture or fibrillation in the same cells. The slow beating rate was returned to the control rate by adding delipidated bovine serum albumin (BSA), which has a high binding affinity for free fatty acids, to the perfusate (not shown). (C) After induction of contracture and fibrillation by toxic concentrations of calcium (5 mM) and ouabain (0. I mM), addition of EPA (8 μm) terminated the fibrillation and contractures and led to slow normal contractions. Subsequent addition of BSA (2 mg/mL) extracted the EPA from the myocytes, and in the continued presence of the high concentrations of calcium and ouabain, reestablished the contractures and fibrillations.

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Abbreviations: AA, arachidonic acid; BSA, bovine serum albumin; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid; VF, ventricular fibrillation.

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taneously and rhythmically so their beating rate and contraction amplitude can be readily recorded. A number of substances known to produce malignant ventricular arrhythmias in humans have been tested for their effects on these isolated myocytes: ouabain (100 μM) or elevated medium calcium concentration (7–8 μM) (7), β-adrenergic agent (isoproterenol, 2–4 μM) (8), lysophosphatidyl choline or acylcarnitine (2–5 μM) (9). When EPA or DHA (2–10 μM) are added to the fluid bathing the myocytes, the beating rate invariably slows within a couple of minutes (Fig. 1). Addition of the EPA or DHA prior to the arrhythmogenic agent will prevent the occurrence of the arrhythmia. But addition in the presence of the contracture and fibrillation induced by the arrhythmic agent will terminate the arrhythmia. Subsequently, the free fatty acids can be extracted from the myocytes by adding delipidated bovine serum albumin (BSA) to the medium perfusing the cells and in 2–3 min the arrhythmia recurs. Albumin has some three high affinity and four lower affinity binding sites for free fatty acids (10). Delipidated albumin can reverse the direction of the usual traffic of free fatty acids and withdraw them from the cells. If instead of the free fatty acid the triglyceride or ethyl ester of EPA or DHA is added to the medium, no prevention or termination of an arrhythmia is achieved. Only the free acid form of the fatty acids is able to prevent or to terminate an arrhythmia promptly. If the EPA or DHA had been ingested orally as the triglyceride, the usual form in which it is present in fish oil, there would have been ample time for esterases in the gut and plasma to liberate the free fatty acid which then would be available to prevent or terminate an arrhythmia.

This simple observation tells us some very important facts about the system. Firstly, only the free fatty acids are antiarrhythmic. Secondly, incorporation of the fatty acid into the phospholipids of the cell membrane or other covalent binding to membrane constituents is not required to produce their antiarrhythmic action; were such the case, the arrhythmia would not recur when the free fatty acid is recalled from the myocytes since it is only the free fatty acid that is avidly bound by the albumin.

With the invariable effect of EPA and DHA to slow the beating rate of the isolated neonatal rat cardiac myocytes without affecting the amplitude of the contractions as a simple assay for antiarrhythmic activity, we screened other fatty acids for their antiarrhythmic potential (7). We found that antiarrhythmic activity was not confined only to EPA and DHA, but included α-linolenic acid (C18:n-3, LNA), the parent compound of the n-3 class of PUFA which can be elongated in our bodies to EPA and DHA. A surprise was that the long chain n-6 PUFAs also had antiarrhythmic capabilities, including linoleic acid (C18:2n-6, LN), the parent fatty acid of the n-6 family from which arachidonic acid can be formed in our bodies. Arachidonic acid proved anomalous in its behavior, however. In one third of the experiments, it produced effects identical to that of EPA and DHA, in another third it had no effect on the beating rate, but in the remaining one third it induced violent contractures and arrhythmias. When the arachidonic acid (C20:4n-6, AA) was added to the myocytes together with inhibitors of cyclooxygenase and lipoxygenase, it invariably then also behaved like EPA and DHA in the assay system. Thus, it is the cyclooxygenase and lipoxygenase metabolites of the AA which are arrhythmogenic and the free fatty acid of AA is antiarrhythmic. Oleic acid (C18:1n-9), a monounsaturated fatty acid, and the saturated fatty acids lauric, myristic, palmitic and stearic had no antiarrhythmic properties. The fatty acids mentioned have all been tested separately for their true antiarrhythmic action in addition to the assay system.

Thus, it seems now that the structural requirement for an antiarrhythmic action is a long-chain, hydrophobic, aliphatic compound with a free carboxylic acid group at one end and two or more carbon-carbon unsaturated bonds. We have not tested rigorously the possibility of different potencies of antiarrhythmic effects among the different PUFA, but EPA and DHA are indistinguishable in their effects and seem to be somewhat more active than other PUFA. Because of the potential arrhythmic actions of eicosanoids generated from AA when n-6 PUFA of vegetable origin are ingested, the n-3 PUFA, which are present in our diets largely from fish oils, rather than the n-6 PUFA are the preferred class of fatty acids to consider for potential clinical application.

Because the EPA and DHA are capable of preventing or terminating tachyarrhythmias caused by diverse arrhythmogenic agents, it became apparent that their action is likely to be on the basic excitability/automaticity of the cardiac myocytes. Thus with Dr. Yong-Fu Xiao we undertook to perform whole cell voltage-clamp measurements to examine the electrophysiologic effects of these PUFA on the electrical cycle of the neonatal rat cardiac myocytes. The results are of considerable interest (11). With one microelectrode within the myocyte and the other in the surrounding bathing medium, the cell membrane electrical potential can be monitored. In the resting or diastolic state, the membrane potential of these cells is some −70 mV. A depolarizing electrical stimulus elicits a classic action potential when the threshold potential for opening of the fast sodium channel is reached and an action potential very similar to that observed in human endocardial ventricular myocytes is elicited. When the isolated myocyte is exposed to EPA or DHA (5–10, μM) added to the bathing medium, the resting or diastolic membrane potential becomes slightly, but significantly, hyperpolarized and the threshold potential for the gating of the sodium channel becomes more positive. The consequence of these two effects is that it now requires a 50% increase in the strength of a depolarizing electrical stimulus to elicit an action potential. Perhaps of even greater importance to the antiarrhythmic action of the PUFA is the marked 150% increase in duration of the refractory period of the myocytes in the presence of the PUFA due to a slowing of the Phase 4 depolarization. The changes in electrophysiology induced by the PUFA are just the opposite of those produced by ischemia which causes partial depolarization of the membrane potential with increased electrical excitability. These changes result from changes in conductances

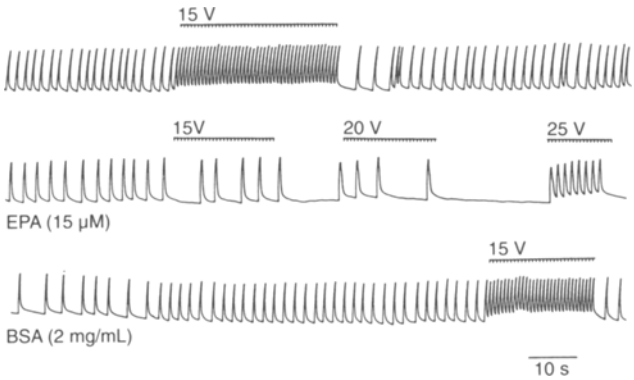


FIG. 2. Effect of EPA on the response of cultured myocytes to electrical pacing. Cells were paced as described in the text. Tracings show the response of cell contractions to the electrical stimuli (as indicated by the bars above the contraction tracing) before perfusion with EPA (top tracing), during perfusion with EPA (15 μm) (middle tracing) and after washout with BSA. In the presence of EPA, the stimuli at external field strength of 15 or 20 volts no longer elicited contraction responses from the myocytes. At a field strength of 25 volts the myocytes begin to respond, but are captured only by every other imposed stimulus. After the EPA has been withdrawn from the myocytes by the BSA, the cells again respond to stimuli at 15 volts field strength. The three tracings were made consecutively from the same cell. Abbreviations as in Figure 1.

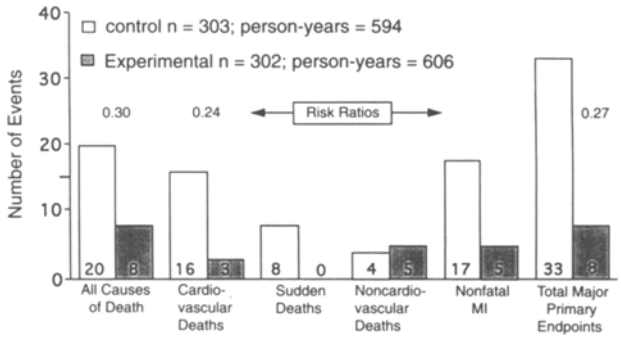


FIG. 3. A summary of the clinical trial, "Mediterranean α-Linolenic Acid-Rich Diet in Secondary Prevention of Coronary Heart Disease" (Ref. 14). 605 men who had a recent heart attack were randomized equally into a control and experimental cohort. The former were instructed to follow a prudent post-infarction diet while the latter were given more stringent dietary instructions, which included omitting red meats and dairy fat and eating more vegetables, fruits, legumes, and breads. They were allowed to use olive oil and given a soft margarine made of canola oil, which contains some 10% α-linolenic acid. Figure 3 summarizes the main results of the study. Relevant to the present topic was the suppression of sudden death among the experimental cohort (with permission of publisher and authors). End points (27 mon mean follow up).

of ion channels in the cell membranes and account for the diminished excitability of the myocytes. The fast sodium channel we have found to be partially inhibited by the PUFA (12), but the reduced duration of the action potential and the hyperpolarization of the diastolic membrane potential suggests that other channels, probably calcium or potassium channels, or even chloride channels, are also modulated by the PUFA.

The decrease in electrical excitability of the myocytes resulting from these electrophysiologic actions of the PUFA can be demonstrated directly, as shown in Figure 2. In this experiment, the myocytes were induced to contract by electrical stimuli generated by an imposed electrical field established between two platinum electrodes. When the electrical field strength was 15 volts, the myocytes increased their beating rate to match the rate of the imposed electrical stimuli. While the myocytes were exposed to EPA (15 μM) stimuli at 15 volts or even 20 volts failed to pace the contractions. At an imposed electrical field strength of 25 volts the cells began to be captured by the stimuli, but responded only to every other stimulus. When the EPA was extracted from the same myocytes by delipidated BSA, the myocytes regained their initial electrical sensitivity and contracted in response to each stimulus imposed at a field strength of 15 volts.

One can readily sense that with this increased stabilization of the membrane potentials in the presence of these fatty acids, the myocytes are less prone to respond to every aberrant depolarizing stimulus which may impinge upon them during an ischemic stress, whether it be from reentry, circus stimuli or from early or delayed after potentials. The slowing of the beating rate of the myocytes is not the cause of the antiarrhythmic properties of the PUFA. It is these electrophysiologic changes induced by the PUFA which result in both their antiarrhythmic effects and the slowing of the beating rate.

Thus far, we have not conducted clinical trials to test the antiarrhythmic effects of these PUFA in humans. There are, however, two published clinical trials, which perhaps inadvertently have examined this possibility; both were secondary prevention trials (13,14). In both studies, striking reductions in all-cause and cardiac mortality were found in the experimental groups, who were receiving the n-3 PUFA. Prevention of cardiac sudden death contributed to the favorable outcomes. Figure 3 is a summary of the more recent of these two clinical trials (14). There was some 70% reduction in all-cause mortality resulting from the reduction in cardiovascular deaths. Noteworthy to this discussion was the prevention of "sudden deaths" in the group receiving α–linolenic acid in their diets. The survival curves in both studies (not shown) demonstrated separation of the mortality for the experimental versus the control cohorts commencing very early (within only a few months) with implementation of the experimental diets. This is in contrast to the findings when cholesterol-lowering treatments were studied, in which a full two years of the regimen transpired before the survival curves separated (15)

Clearly, we have much more work ahead to determine exactly which ion channels are affected by the PUFA and to learn how the PUFA change the conductances of the ion channels. However, we feel now that with the modest understanding we have already gained and the unquestionable prevention of ischemia-induced malignant ventricular arrhythmias in our dog studies (6), which support the findings in feeding studies in experimental animals or *ex vivo* tissue studies by others (2,3–5,16), it is time that carefully designed clinical trials be undertaken to test the antiarrhythmic effects of these

PUFA in humans. Each year in the United States alone some 50 to 60% of the 500,000 deaths annually resulting from acute myocardial infarctions are due to malignant ventricular arrhythmias occurring within one hour of the acute infarction so the potential benefit from these to n-3 PUFA of fish oil may be large.

ACKNOWLEDGMENTS

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Eicosapentaenoic Acid Is Primarily Responsible for Hypotriglyceridemic Effect of Fish Oil in Humans

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ABSTRACT: The aim of this study was to determine whether eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA), or both, were responsible for the triglyceride (TG)-lowering effects of fish oil. EPA (91% pure) and DHA (83% pure), a fish oil concentrate (FOC; 41% EPA and 23% DHA) and an olive oil (OO) placebo (all ethyl esters) were tested. A total of 49 normolipidemic subjects participated. Each subject was given placebo for 2–3 wk and one of the n-3 supplements for 3 wk in randomized, blinded trials. The target n-3 fatty acid (FA) intake was 3 g/day in all studies. Blood samples were drawn twice at the end of each supplementation phase and analyzed for lipids, lipoproteins, and phospholipid FA composition. In all groups, the phospholipid FA composition changed to reflect the n-3 FA given. On DHA supplementation, EPA levels increased to a small but significant extent, suggesting that some retroconversion may have occurred. EPA supplementation did not raise DHA levels, however. FOC and EPA produced significant decreases in both TG and very low density lipoprotein (VLDL) cholesterol (C) levels ($P < 0.01$) and increases in low density lipoprotein (LDL) cholesterol levels ($P < 0.05$). DHA supplementation did not affect cholesterol, triglyceride, VLDL, LDL, or high density lipoprotein (HDL) levels, but it did cause a significant increase in the HDL_2/HDL_3 cholesterol ratio. We conclude that EPA appears to be primarily responsible for TG-lowering (and LDL-C raising) effects of fish oil.
Lipids 31, S-45–S-49 (1996)

It has long been known that fish oil lowers triglyceride levels by about 25–35% (1), and it has been assumed that eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), or both were responsible. Few controlled studies in humans, however, have examined EPA and DHA supplementation separately to determine effects of each fatty acid (FA). Supplementation with purified EPA has in most studies lowered triglyceride levels, but these have not been well-controlled trials (2–9). Effects of purified DHA on human serum lipoprotein levels has not been reported. The purpose of placebo-controlled studies reported here was to compare effects of relatively pure EPA and DHA to a fish oil concentrate (FOC) containing both EPA and DHA on fasting lipids, lipoproteins, and phospholipid FA composition in healthy volunteers.

MATERIALS AND METHODS

Subjects. Healthy subjects (n = 49) were recruited from the University of Kansas Medical Center staff and student population. The subjects were normolipidemic, except that triglyceride (TG) levels were in the higher end of normal (50th–90th percentile for their age and sex). Both men and women were included in the study. Characteristics of the study groups are shown in Table 1.

Supplements. All n-3 FA supplements were given as ethyl esters and may be obtained from the Fish Oil Test Materials Program of the U.S. National Institute of Health/Department of Commerce. All supplements were provided in 1-g capsules, except for 14 of the 25 subjects in the EPA group, who were given EPA in liquid form (with the oil dispensed in single-dose, opaque, plastic vials, flushed with N_2 and stored at 4°C); the other 11 subjects received encapsulated EPA. Liquid supplementation was necessary because it was the only dosage form available at the time these subjects were studied. FOC, EPA, and DHA preparations contained the following amounts of EPA and DHA, respectively: 41% and 23%; 91% and 1.7%; and 4.3% and 83%. The OO placebo contained 67% ethyl oleate.

Protocol. Data from three separate studies are reported in this paper. In the first study, 11 subjects were assigned to take 3 grams per day (g/d) EPA, 5 g/d FOC and 5 g/d OO placebo. In the second study, 9 subjects were assigned to take 3 g/d DHA, 5 g/d FOC, and 5 g/d OO placebo. Both of these were randomized, crossover, single-blind studies with at least 2 wk of wash-out between the 3-wk supplementation phases. All supplements given were encapsulated. In the third study (conducted several months later), 29 subjects were randomly assigned to take either 3 g/d EPA in liquid form (n = 14), or 5 g/d FOC (n = 15) in addition to the OO placebo in a parallel-group design. Placebo was taken for 2 wk followed by n-3 FA supplements for 3 wk. In all, a total of 9 subjects took OO and DHA (DHA group), 25 subjects took OO and EPA (EPA group), and 35 subjects took OO and FOC (FOC group). The subjects were instructed to maintain their normal lifestyles

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Abbreviations: C, cholesterol; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; FOC, fish oil concentrate; HDL, high density lipoproteins; LDL, low density lipoproteins; LpL, lipoprotein lipase; OO, olive oil; TG, triglyceride, VLDL, very low density lipoproteins.

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and background diets throughout the study. Fasting blood samples were drawn twice at the end of each supplementation period and analyzed for changes in lipid and lipoprotein levels. FA composition of the plasma phospholipid fraction was also determined.

Methods. Serum total cholesterol, TG, and high density lipoproteins (HDL) cholesterol (C) were measured enzymatically on a Cobras Mira as previously described (10). Very low density lipoprotein (VLDL) and low density lipoprotein (LDL) C levels were calculated by the Friedewald equation (11). Plasma lipids were extracted and separated by thin layer chromatography, and FA composition in the phospholipid fraction was determined by combined thin layer and gas chromatography as previously described (10).

Statistics. Data obtained from the two blood draws at the end of each supplementation phase were averaged before comparison. Data are presented as mean ± SD. Values at the end of placebo and n-3 treatment phases were compared by the paired *t* test, and a difference with $P < 0.05$ was considered to be statistically significant.

RESULTS

Compliance. Compliance was assessed by capsule count and by weighing the EPA vials before and after dosing. More than 95% of each supplement was taken; adjusting for supplement composition, mean intakes (g/d) of EPA and DHA were as follows: FOC, 2.01 and 1.12; EPA, 2.81; and DHA 2.36. Thus total n-3 FA intakes were similar in the three supplementation groups (2.4–3.1 g/d), and intakes of EPA and DHA, when given singly, were greater than those taken by the FOC group.

Plasma phospholipid FA composition. Linoleic and arachidonic acid levels decreased significantly on the FOC and EPA supplements (Table 2). FOC supplementation increased plasma phospholipid EPA by fourfold and DHA by twofold vs. placebo levels. Supplementation with purified EPA led to the same fourfold increase in EPA levels but did not change DHA levels. DHA supplementation more than doubled DHA levels and increased EPA significantly (0.3 to 0.7%).

Fasting lipids and lipoproteins. On FOC supplementation, TG and VLDL cholesterol levels were lowered by 20% ($P < 0.001$; Table 3). FOC also caused the LDL cholesterol level to increase by 10% ($P < 0.05$). Total HDL cholesterol did not change on FOC supplementation, but the HDL_2 cholesterol level increased by 27% ($P < 0.01$) while the HDL_3 cholesterol decreased by 6% ($P < 0.01$). EPA lowered TG and VLDL C by 16% and increased in LDL cholesterol levels by 6% (both $P < 0.01$). Although EPA increased total HDL C level by 6% ($P < 0.05$), there were no significant changes in HDL subfractions. DHA supplementation, on the other hand, had no TG-lowering or LDL-raising effect. The only lipoproteins affected by DHA were HDL subfractions. HDL_2 C increased by 38%, and HDL_3 C decreased by 8% (both $P < 0.05$). Total C level did not change on any supplement.

TABLE 1
Characteristics of Study Groups

Characteristics	EPA[a] group ($n = 25$)	DHA[b] group ($n = 9$)	FOC[c] group ($n = 35$)
Sex (M/F)	8/17	4/5	13/22
Age ± SD	30.1 ± 6.6	31.0 ± 11.8	28.9 ± 7.6
BMI[d] ± SD	26.0 ± 2.9	24.5 ± 2.2	24.2 ± 2.3
Screening lipids (mmol/L)			
Cholesterol	4.80 ± 0.73	4.70 ± 0.61	4.72 ± 0.68
Triglyceride	1.46 ± 0.31	1.40 ± 0.39	1.40 ± 0.47
HDL C[e]	1.21 ± 0.32	1.25 ± 0.31	1.27 ± 0.32
LDL C[f]	2.89 ± 0.74	2.68 ± 0.73	2.73 ± 0.68

[a]EPA = Eicosapentaenoic acid.
[b]DHA = Docosahexaenoic acid.
[c]FOC = Fish oil concentrate.
[d]BMI = Body mass index.
[e]HDL C = High density lipoprotein cholesterol.
[f]LDL C = Low density lipoprotein cholesterol.

DISCUSSION

This is the first study to compare the effects of (relatively) pure EPA and DHA to both a placebo and to a FOC containing both FA in humans. The most well-known effect of the FOC-decreased TG levels was reproduced by supplementation with EPA, but not with DHA. Since the DHA group was relatively small (n = 9 vs. 25 in the EPA group and 35 in the FOC group) and thus underpowered, we cannot be sure that it would not have an effect in a larger group. The failure of DHA to achieve a statistically significant TG reduction, however, was caused by a small mean effect (–3%), not by a large variance. Conversely, the percent change in LDL C was identical for both EPA and DHA (+6%), but the smaller number of subjects in the latter group prevented the difference from being significant. It is possible that combined with EPA, DHA might be more effective. It should be noted that we fed 2.4 g DHA in the DHA period and less than half this amount in the FOC period; yet TG were reduced with FOC but not DHA. Thus EPA is more likely than DHA to be the hypotriglyceridemic agent in fish oils.

Although EPA has been reported in 7 studies to be hypotriglyceridemic in humans (2–8), all were "before-and-after" designs, and none included a placebo comparison. In the only study to include a placebo (9), EPA did not significantly lower TG levels. Previous human studies using purified DHA have not been reported. Childs *et al.* (12) fed EPA-rich and DHA-rich fish oils to normal volunteers in an attempt to determine how each FA affected lipid levels. They concluded that both EPA and DHA were equally hypotriglyceridemic, but that DHA also lowered LDL, while EPA did not. The difficulty with this study was the use of very high intakes of n-3 FA. For example, the DHA-rich diet (15 g DHA/d) was "contaminated" with 5 g EPA—nearly twice the amount of EPA fed here to our EPA group. Likewise, the EPA-rich diet (which provided 12 g EPA/d) contained more DHA (3.1 g/d) than our DHA group received. Thus no conclusions regarding separate effects of EPA and DHA can be drawn from this study.

Effects of EPA and DHA on lipoprotein metabolism have been studied in rats and in cultured cells. EPA has lowered circulating TG levels in rats (13,14), but only with very high doses (1–2 g/kg/d). Adjusted for body surface area, equivalent

TABLE 2
Effects of n-3 Fatty Acid (FA) supplements on Selected FA from the Plasma Phospholipid Fraction (% Total FA)[a]

	Placebo	FOC	Placebo	EPA	Placebo	DHA
Fatty acid	(n = 35)		(n = 25)		(n = 9)	
Oleic acid	10.4 ± 6.5	8.4 ± 2.2	8.6 ± 2.6	7.7 ± 2.3	9.5 ± 1.6	8.7 ± 1.1
Linoleic acid	17.1 ± 4.3	15.2 ± 4.2[b]	16.1 ± 4.9	13.4 ± 3.9[b]	19.8 ± 2.2	16.8 ± 5.7
Arachidonic acid	8.7 ± 3.8	6.7 ± 2.2[b]	7.8 ± 2.8	6.5 ± 2.4[b]	9.1 ± 1.2	8.3 ± 1.7
Eicosapentaenoic acid	0.7 ± 0.5	3.2 ± 1.3[b]	0.9 ± 0.6	4.0 ± 1.7[b]	0.3 ± 0.1	0.7 ± 0.4[b]
Docosahexaenoic acid	2.0 ± 0.6	3.3 ± 1.3[b]	1.7 ± 0.7	1.7 ± 0.6	2.3 ± 0.6	5.2 ± 0.9[b]

[a]See Table 1 for abbrevations.
[b]$P < 0.005$ vs. respective placebo.

doses in humans would be 12–24 g/d (15). We fed less than 3 g/d in this trial and found a clear hypotriglyceridemic effect. DHA was without hypotriglyceridemic effect in rats even at these high doses (13,14). Studies in cultured cells and perfused livers have shown that EPA inhibits TG synthesis/secretion in rat (16,17) and rabbit (18) hepatocytes, in $HepG_2$ cells (19,20), and in perfused rat livers (21,22). These findings are consistent with our observations in humans. DHA, however, was also reported to lower TG secretion from rat hepatocytes (23), $HepG_2$ cells (19), and perfused livers (21), and these findings contradict human and intact animal data.

In most studies in normolipidemic subjects, n-3 FA have had no significant impact on LDL C levels, but, in hypertriglyceridemic patients, LDL usually increases with fish oil feeding (1). Reasons for this increase have never been clearly determined. If fish oil inhibits hepatic TG production, then it is difficult to explain why LDL (the product of VLDL catabolism) levels are increased. If precursor (VLDL) levels are reduced, then product (LDL) levels should be, also. It has been suggested that fish oil feeding reduces the size of VLDL secreted by the liver (24), and further that these smaller VLDL particles are more readily converted into LDL (25). There is evidence, however, to suggest that n-3 FA do not increase circulating levels of small VLDL (26). Another mechanism by which n-3 FA might raise LDL levels is by stimulating the conversion of VLDL to LDL by increasing lipolytic rates *in vivo*. This seems unlikely, since fish oil treatment has repeatedly been shown to not stimulate lipoprotein lipase (LpL) activity measured in postheparin plasma both in humans (27–29) and in animals (30–32). In addition, intralipid clearance is not enhanced by pretreatment with n-3 FA (10). Conversely, n-3 FA supplementation stimulated endogenous (preheparin) LpL activity (33), although these findings await confirmation. The other possible mechanism for the LDL-raising effect of n-3 FA is slowed LDL catabolism, perhaps due to an inhibition of LDL receptor activity, as has been suggested from cell culture studies (20,34,35) but not in intact rats (36). There is no good human data regarding this question.

Both FOC and DHA significantly increased HDL_2 C while decreasing HDL_3 C levels. A similar effect has been reported with fish oil feeding in normal volunteers (37–39) and in hyperlipidemic patients (40,41). EPA alone had no such effect (3) in confirmation of our findings here. HDL_2 C levels have been inversely associated with coronary heart disease in seven of eight recent studies and with the severity of coronary disease in six of nine studies (42). Thus n-3 FA-induced increase in this HDL subfraction may contribute to the antiatherogenic potential of fish oils.

We were able to explore the question of how EPA and DHA are interconverted in adult humans. The underlying assumption has long been that EPA is elongated and desaturated directly to DHA (43). Sprecher and colleagues (43), however, have now shown in the rat that, although EPA can be converted into DHA, it takes a more circuitous route, being first converted into 24:6n-3 and then β-oxidized back to 22:6n-3. Whether this pathway exists in adult humans is not known, but since high intakes of 18:3n-3 (linolenic acid) can produce small increases in both EPA and DHA levels (44–46), it is assumed to be active. Results of our study argue against direct conversion of EPA to DHA and somewhat in favor of retroconversion of DHA to EPA. von Schacky and Weber (47) reported that 6 d of 6 g EPA/d resulted in no net increase of DHA in plasma phospholipids. They also found that DHA feeding (same dose and duration) increased EPA levels in the same lipid class. Retroconversion of DHA to EPA has also been reported by others (7). Findings of our study using many

TABLE 3
Effect of n-3 Supplements on Lipid and Lipoprotein Levels (mmol/L)[a]

	OO[b]	FOC	OO	EPA	OO	DHA
Lipid/lipoprotein	(n = 35)		(n = 25)		(n = 9)	
Cholesterol	4.49 ± .69	4.54 ± .79	4.63 ± 67	4.74 ± .77	4.31 ± .65	4.46 ± .67
Triglyceride	1.32 ± .45	1.05 ± .35[c]	1.38 ± .46	1.17 ± .41[c]	1.22 ± .31	1.17 ± .38
VLDL C	0.61 ± .21	0.48 ± .16[c]	0.63 ± .21	0.53 ± .19[c]	0.56 ± .14	0.53 ± .17
LDL C	2.60 ± .66	2.74 ± .74[c]	2.81 ± .65	2.97 ± .77[c]	2.56 ± .61	2.72 ± .64
HDL C	1.34 ± .34	1.38 ± .38	1.17 ± .31	1.24 ± .34[c]	1.18 ± .31	1.20 ± .35
HDL_3 C	1.00 ± .21	0.94 ± .18[c]	0.87 ± .25	0.90 ± .18	0.95 ± .22	0.87 ± .15[c]
HDL_2 C	0.34 ± .18	0.43 ± .25[c]	0.30 ± .20	0.34 ± .20	0.24 ± .10	0.33 ± .20[c]

[a]See Table 1 for abbreviations.
[b]OO = Olive oil.
[c]Significantly different at least $P < 0.05$.

more subjects fed smaller doses of n-3 FA for 21 d instead of 6 d support these conclusions. Another possible explanation of our findings, however, is the small amount of EPA in the DHA supplement (4%; providing 120 mg EPA/d) may have contributed to the small rise in plasma EPA levels (Table 2). Thus, from our data, no firm conclusion can be made *vis à vis* retroconversion of DHA to EPA.

In conclusion, we have obtained evidence that EPA is likely to be the hypotriglyceridemic FA in fish oils and that it is not readily converted into DHA. Furthermore, although DHA has little to no impact on serum lipid levels, it may be retroconverted to EPA in humans. Further studies are needed to clearly define individual effects of EPA and DHA on human lipid metabolism.

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A New Relationship Between Total/High Density Lipoprotein Cholesterol and Polyunsaturated Fatty Acids

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ABSTRACT: Dietary and plasma fatty acids have been linked to total cholesterol but not to the ratio of total/high-density lipoprotein cholesterol (TC/HDLC). To evaluate the relationship between dietary and plasma levels of polyunsaturated fatty acids (PUFA) and TC/HDLC, we analyzed cross-sectional and longitudinal data using 519 plasma samples (50% men, 50% women) from subjects participating in the Framingham Heart Study and results from a study feeding diets rich in either n-6 linoleic acid or n-3 α-linolenic acid with or without fish oil supplements (n-3 derivatives). Values of TC/HDLC are inversely related to the percent of plasma PUFA when both variables are measured at the same time in different subjects, $R = 0.82$, $P < 0.000001$. PUFA in phospholipids increase in response to increased dietary intake of different PUFA, either n-3 or n-6 or fish oils. There was a highly significant inverse relationship between TC/HDLC and the percent of PUFA in phospholipids, $R = 0.97$, $P < 0.001$. The relationship was similar regardless of the source and type of dietary fatty acids. A similar relationship existed when only the baseline points were considered. When plasma PUFA % increases, either in response to a diet high in PUFA or across different subjects, TC/HDLC ratios decline. Evaluation of plasma fatty acid profiles and increased balanced dietary intake of PUFA to bring fatty acid profiles of subjects with low PUFA plasma levels closer to the profile of a healthy reference group is an effective approach to reduce high TC/HDLC. Reductions of more than 50% in TC/HDLC appear feasible with dietary modification alone. Further research into fatty acid metabolic activity may determine the biochemical basis of common dyslipidemias.
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It is well established that increased dietary polyunsaturated fatty acids (PUFA) reduce total/high density lipoprotein cholesterol (TC/HDLC) (1,2). Dietary intervention studies provided evidence that eating PUFA and fish oils reduces biochemical risk factors associated with cardiovascular disease (3). Optimization of lipoprotein levels with diet or drugs regresses coronary artery disease (4) and reduces the risk of angiographic progression (5), a risk reduction attributed primarily to a reduction in TC/HDLC (American Heart Association, 1995 Annual Meeting, unpublished communications).

It is not known whether substantial reductions in TC/HDLC, similar to those achieved with drugs, can be achieved with the essential fatty acids (EFA) linoleic acid (18:2n-6, LA), α-linolenic acid (18:3n-3, ALA) or fatty acids found in fish oil (ALA n-3 derivatives). A computer search found no published data on the relationships between plasma fatty acids and TC/HDLC levels, although many equations have been described linking dietary fatty acids to cholesterol. The problem with dietary data alone is that it is impossible or impractical to accurately track dietary fatty acids, conversion of calories to PUFA or other fats, calorie utilization, and metabolism. Thus dietary intake data are only a rough approximation of what people eat and accumulate as PUFA, even when subjects comply with experimental requirements.

Since the 1960s, Kingsbury *et al.*, among others, found fatty acid abnormalities in patients with different types of atherosclerosis (6) and myocardial infarction (7). Serum fatty acid patterns were shown to be good predictors of myocardial infarction (8), and low dietary intake of LA correlated with the predisposition to myocardial infarction (9). Moreover, among several variables, LA in serum total lipids was found to be the single most effective predictor of cardiovascular death in postinfarction middle-aged men (10). Lower levels of PUFA were found in phospholipids of subjects with sudden cardiac death (11). In addition to preventing or correcting physiological abnormalities that may arise from low plasma PUFA levels, correction of abnormally low levels of PUFA with dietary PUFA has additional clinical applications if it can be shown to reduce cardiovascular end-points (such as myocardial infarct, angina, stroke, and hypertension). Using food to reduce the TC/HDLC ratio, considered one of the best surrogate indices for heart disease risk, would have implications for health care costs. Lowering TC/HDLC with diet or drugs can prevent heart disease morbidity and mortality and regress atherosclerotic disease. Lowering TC/HDLC with drugs can cost more than $2,000 per patient per year. With

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Abbreviations: ALA, α-linolenic acid; EFA, essential fatty acids; EFAD, essential fatty acid deficiency; EFAI, essential fatty acid insufficiency; LA, linoleic acid; PUFA, polyunsaturated fatty acids; TC/HDLC, total/high density lipoprotein cholesterol.

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more than 50,000,000 Americans having abnormal cholesterol, treating everybody with drugs would cost more than $100 billion annually!

In a cross-sectional study of about 100 subjects, criticized as having a small sample size, Siguel and Lerman (12) found an inverse relationship between PUFA and TC/HDLC. To confirm and expand those results, we report here the analyses of 519 samples from the Framingham Heart Study. In a recent study, Mantzioris *et al.* (13) fed 30 subjects n-3 or n-6 fatty acid–rich diets. They found what appeared to be a "neutral effect on cholesterol." We analyzed both sets of data using a different model to characterize fatty acid status and found that increasing low PUFA levels with dietary PUFA drastically decrease TC/HDLC in the manner we predicted (12). These relationships have major implications for nutrition policy and medical care.

MATERIALS AND METHODS

We obtained about 3,000 leftover plasma specimens kept frozen for 3–6 years at –70°C from participants in the Framingham Cardiovascular Offspring Study Cycle 4. Fatty acids were measured using 0.100 mL plasma (14,15) , the extraction procedure described by Lepage and Roy (16), and a 100 m capillary column optimized to separate fatty acids over approximately 180 min. We identified peaks that characterize sample deterioration. There was no evidence of fatty acid degradation in the samples used. Any deterioration would most likely reduce the PUFA, introducing errors that lower the statistical significance of the models used. We selected for fatty acid analyses 519 samples randomly distributed by subject age and sex (ages 30–69, approximately 50% men and 50% women). Our objective was to have 480 samples; we oversampled, anticipating that some of the samples would have deteriorated. No evidence of deterioration was seen, and therefore all were used. To eliminate unusual subjects with rare metabolic disorders, we restricted this study to subjects with cholesterol between 90 mg/dL and 350 mg/dL and subjects with triglycerides <400 mg/dL.

Many factors, such as pipetting, evaporation, calibration, venipuncture method, fasting, and recent diet, can increase or decrease total and HDL cholesterol measurement. The ratio TC/HDLC cancels some of this variability due to sample concentration, dilution, or other factors that proportionately affect both variables. For these reasons, TC/HDLC may be a more reliable variable than total or HDL cholesterol alone. Among the fatty acid variables, PUFA (%) summarizes all the EFA and their polyunsaturate derivatives. Because PUFA concentration in mg/dL of plasma is highly correlated with lipid levels, whole plasma fatty acid concentrations is not a suitable independent variable (12). Most plasma PUFA is found in lipoproteins. Thus a reasonable biological hypothesis is that the concentration of PUFA in lipoproteins, roughly equivalent to the percentage of PUFA in plasma, is a major variable associated with TC/HDLC.

Statistical significance usually tests for difference from zero. Many variables have regression coefficients with values slightly larger than zero, which are statistically different from zero with a large sample size. To confirm that PUFA was strongly associated with TC/HDLC, we used a technique that further reduced the effects of weak relationships.

Samples were sorted by sex and ordered in accordance with the previously measured values of TC/HDLC. Rather than using the more conventional approach of dividing the sample into tertiles or quartiles (3–4 groups), we computed average values of 10 consecutive samples for a total of 52 data points (25 men, 27 women, one of the points had 9 cases). This averaging process eliminates a spurious relationship between TC/HDLC and PUFA and reduces random variability and measurement error. This approach is rarely used because many relationships in epidemiological studies are weak and would disappear. We averaged values of 10 data points to preserve strong relationships, while eliminating as much as possible of random variability and weak relationships. In general, grouping more data points eliminates more weak variables and leaves fewer points for further multivariate analysis. Groups of 10 were chosen as a reasonable compromise for future analysis. Averaging reduces effects of variables that are randomly distributed across, i.e., are independent of, TC/HDLC. If there is no relationship between TC/HDLC and PUFA plasma levels, the distribution of PUFA levels is similar for all groups of subjects. If PUFA is a poor predictor of TC/HDLC, average values of PUFA for each group would be about the same, and the R value of the equation linking PUFA to TC/HDLC would be small. This approach detects biologically significant differences rather than merely statistically significant differences found with a large sample size. The nature of the relationship is not altered by grouping the data.

Reference (normal) levels for EFA were determined in the manner usually performed with other essential nutrients. Individuals without known disease and with biochemical parameters within established normal levels (healthy subjects) were used to establish reference ranges. We diagnose EFA deficiency (EFAD) or insufficiency (EFAI) by biochemical deviation from established reference ranges in the same manner that we diagnose low vitamin or mineral levels, low HDL, or high cholesterol (18). The term EFAI was created to distinguish biochemical EFAD from EFAD previously associated with severe clinical signs.

Mantzioris *et al.* (13) randomly assigned 30 subjects to two dietary treatment groups. One group took flaxseed oil rich in ALA (n-3) for 4 wk, supplemented later by 4 wk on fish oil. The other group followed a diet rich in LA (n-6) for 4 wk, supplemented later by 4 wk on fish oil. Fatty acids in plasma phospholipids were measured at baseline, 4 wk and 8 wk, for a total of 6 measurements. Although the range of PUFA values was narrower for phospholipids than for whole plasma, consistent with the hypothesis that humans closely regulate PUFA phospholipid levels, there was a positive linear relationship between PUFA % levels in plasma and phospholipid (Siguel, E., unpublished data). We searched for relationships relating the percent or ratio of lipid variables to the percent or ratio of

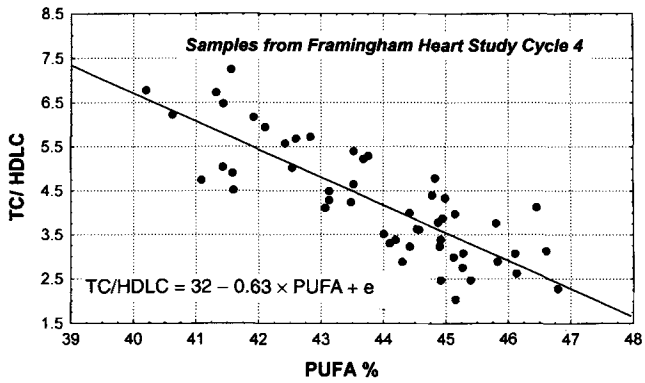


FIG. 1. Changes in total/high density lipoprotein cholesterol (TC/HDLC) vs. plasma polyunsaturated fatty acids (PUFA).

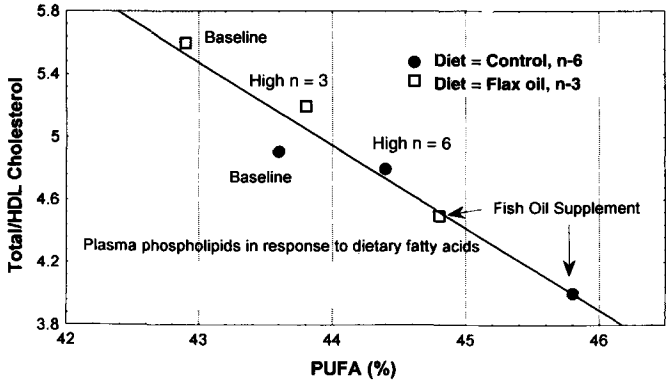


FIG. 2. Changes in total/high density lipoprotein cholesterol vs. increase in n-6 and n-3 fatty acids. See Ref. 13.

fatty acid variables, particularly the ratio of TC/HDLC as a function of PUFA (%). Statistical and graphics analyses were done using EXCEL (Microsoft Corp., v. 5.0); and STATISTICA (StatSoft, Inc., Tulsa, OK).

RESULTS

Regression analysis with all 519 points found a highly significant statistical relationship with PUFA. However, such relationship could be a statistical artifact. Linear regression using the 519 data points with TC/HDLC as the dependent and PUFA (%) as the independent variables had $R = 0.32$ ($F = 58$, $P < 0.000001$). Limiting the analysis to cholesterol values between 100 and 300 and triglycerides under 300 eliminated 16 samples, with $R = 0.27$ ($F = 39$, $P < 0.000001$). Figure 1 displays the relationship between TC/HDLC and PUFA (in percent) for the 52 groups of subjects. The relationship is highly linear, with $R = 0.82$, $P < 0.000001$. Either with the grouped or individual data, TC/HDLC declines as PUFA (%) increases.

Table 1 lists the values of TC/HDLC, PUFA, monounsaturated fatty acids (MUFA), PUFA/MUFA, and PUFA/saturated and monounsaturated fatty acids (NoPUFA) for each of the 6 data points (3 diets or 3 time periods for n-3 and n-6) in the Mantzioris *et al.* study (13). At baseline, subjects had PUFA levels below our reference values (18), suggesting that these subjects had EFAI according to our criteria (12). As expected, plasma PUFA increased when subjects ate more dietary PUFA, either in the form of n-3 or n-6 fatty acids or fish oil. Figure 2 shows the inverse relationship between plasma TC/HDLC and PUFA, correlation coefficient $R = 0.97$, $P < 0.001$.

DISCUSSION

These results show that in cross-sectional (different subjects, one time blood measurements) and longitudinal studies (same subjects on different diets for different time periods), TC/HDLC declines when PUFA increases, in a direct and highly linear inverse proportion to PUFA levels. Together, these results provide evidence that plasma PUFA levels have a significant effect on TC/HDLC and that TC/HDLC levels can be modulated by changes in dietary PUFA. A related study feeding diets low or high in fat and high in PUFA found similar relationships, with those subjects eating the low-fat diet having higher TC/HDLC and lower PUFA than subjects eating a high PUFA diet (19). Although not shown, both the percent and concentration of saturated and monounsaturated fatty acids are positively correlated with TC/HDLC in the Framingham samples. Future reports will describe relationships between individual lipids, percents and concentrations of specific fatty acids, age, sex, height, and weight.

Mantzioris *et al.* (13) reported a different model of the relationship between cholesterol and fatty acid profiles. Instead of comparing TC/HDLC, the authors compared total and HDL cholesterol. Comparing only changes in cholesterol across treatment groups, as done by the previous authors, is ineffective because individuals rarely follow the treatment diet as well as the investigators expect, and thus the expected group differences due to diet may not materialize when gauged by plasma PUFA. Differences in exercise, environmental temperature, body metabolism, and eating behavior drastically affect the net balance of PUFA in the body.

TABLE 1
Cholesterol Ratios vs. Fatty Acid Ratios

Case	Code	Weeks	TC/HDLC[a]	PUFA[b]	MUFA[c]	PUFA/MUFA	PUFA/NoPUFA[d]
Baseline	Control	0	4.91	43.6	11.8	3.69	0.77
n-6	Control	4	4.80	44.4	11.6	3.83	0.80
+ Fish oil	Control	8	4.00	45.8	10.0	4.58	0.85
Baseline	Flax	0	5.60	42.9	12.2	3.52	0.75
n-3	Flax	4	5.20	43.8	11.2	3.91	0.78
+ Fish oil	Flax	8	4.50	44.8	10.2	4.39	0.81

[a]T/HDLC = Total/high density lipoprotein cholesterol.
[b]PUFA = Polyunsaturated fatty acids.
[c]MUFA = Monounsaturated fatty acids.
[d]NoPUFA = Saturated and monounsaturated fatty acids.

Blood lipid values are difficult to predict from dietary intake because the same diet will produce different changes in body fatty acid composition in different individuals (it is impossible to have identical lifestyles, and so forth). Studies that evaluate the risk-protecting effects of different mixtures of dietary fatty acids or low-fat diets ought to analyze the plasma fatty acid composition of subjects to determine the net effects of carbohydrate and fat and their interaction with exercise, food processing (i.e., hydrogenation), oxidation, and other variables affecting net *cis* EFA intake. Dietary intake data may misrepresent what the subject actually eats. For example, n-3 intake is almost impossible to ascertain from dietary data because of the huge variability in n-3 composition of oils used with foods even when the same brand is used (due to factors such as hydrogenation and cooking) and the lack of data on foods rich in n-3 such as green peas and eggs. There are no requirements that food manufacturers maintain constant EFA composition even in their own brands; EFA composition of common foods such as eggs or chicken varies according to the feed used, which in turn depends on global market prices for corn (no n-3), soy (has n-3), and other chicken feeds.

Thus it is hazardous to explain physiological changes only on the basis of presumed dietary intake; it is essential to do plasma fatty acid analysis to obtain a better gauge of changes in body fatty acid composition. Plotting TC/HDLC vs. plasma PUFA values relates two physiological variables measured on the same sample and can be a better indicator of the effects of dietary fatty acids on plasma lipids.

What is noteworthy is that the % of plasma PUFA accounts for most of the variability in TC/HDLC, as can be seen from Figures 1 and 2. These findings support the hypotheses of Siguel and Lerman (12) and Sinclair (24) that the level of PUFA is the most important factor in atherosclerotic and coronary heart disease. As can be seen in Table 1, HDL increases and triglycerides decrease when PUFA increases, in the manner predicted by Siguel and Lerman (12), who proposed that plasma cholesterol and triglyceride levels are determined or regulated, in great part, by EFA metabolism, which is in part determined by the total amounts of each type of fatty acid in the body.

We hypothesize that low-calorie diets reduce TC/HDLC in many overweight subjects by burning saturated fatty acids preferentially over PUFA and thereby increasing the percent of total plasma PUFA. This effect is most pronounced when overweight subjects with significant adipose tissue reserves of PUFA lose weight. When normal weight individuals maintain their weight on low-calorie, low-fat diets low in EFA, however, plasma PUFA is likely to decline, and TC/HDLC may increase (19,27). Replacing fat with calories from low-fat foods may decrease plasma PUFA levels and therefore increase TC/HDLC. Consistent with this hypothesis, a study by Schaefer *et al.* (28) found that low-fat diets, without weight loss, lead to a substantial decline in HDL, and increases in triglycerides and TC/HDLC. During their study (about 3 mon), 2/29 subjects (about 6%) developed evidence of heart disease; one had an infarct.

There is overwhelming evidence that diets rich in PUFA can lower TC/HDLC. PUFA, particularly n-3 derivatives, have been proposed for the treatment of a variety of cardiovascular diseases (29). The active conversion of EFA precursors, ALA and LA, to their derivatives (14) suggests that many people obtain adequate n-3 and n-6 derivatives from ALA and LA, and that these fatty acids can produce drastic declines in TC/HDLC. Subjects with low body levels of EFA or those who need EFA derivatives could be at high risk on a low-fat or strict vegetarian diet deprived of EFA derivatives. Reduced formation of EFA derivatives has been associated with insulin-dependent diabetes, alcoholism, and aging (30). For these subjects, a low-fat diet or a vegetarian diet deprived of EFA derivatives could lead to higher TC/HDLC, higher triglycerides, and perhaps an increased incidence of heart disease. These results do not mean that all PUFA have equivalent effects on TC/HDLC. The subjects in these feeding studies had substantially elevated TC/HDLC ratios. They also had substantially lower then desirable levels of PUFA. When both n-6 and n-3 levels are low, most mixtures of n-6 and n-3 may decrease TC/HDLC. The effects of different n-6 and n-3 fatty acid mixtures are likely to be different for subjects less deficient in PUFA.

It is generally accepted that proper dietary treatment requires a mixture of n-3 and n-6 fatty acids and their derivatives because ideal health depends on an optimal ratio of n-3/n-6 fatty acids (31). Siguel and Lerman (12) recommend a treatment diet that will bring the plasma fatty acid profile of a patient closer to the profile of a healthy reference population. A trial period of ingestion of an oil high in both 18:2n-6 and 18:3n-3 (i.e., soybean oil), together with appropriate antioxidants, may be the best way to raise EFA and decrease cholesterol. After soybean oil supplementation for several months, fatty acid analysis helps to determine whether 18:3n-3 is sufficient to increase 20:5n-3 in blood and lower triglycerides, or whether fish oils are necessary to lower triglycerides. One must also recognize that the dietary balance of n-3 and n-6 can be a modulator of eicosanoid activity, platelet aggregation (3), and incorporation of PUFA in cell membranes (19).

Linear regression is a customary statistical method, and it is easy to interpret, but its value is limited to the range analyzed. Averaging TC/HDLC ratios reduces the influence of extreme points and narrows the range of PUFA %. Individuals can have plasma values of PUFA below 39% or above 48%, not shown in Figure 1. Obviously, Figure 1 does not imply that when PUFA = 60%, TC/HDLC = 0. The biological relationship between TC/HDLC and PUFA % most likely follows a curve that would plateau at TC/HDLC slightly below 2; finding a more biological relationship requires further studies.

Individual patients have a great deal of biological noise in their measurements, and they may not respond to maneuvers that seem very plausible on a population basis. While, on a statistical basis, increasing PUFA may lower TC/HDLC, on an individual basis results are less predictable. Physiological and measurement variability in cholesterol and PUFA % may exceed 30%. Thus the specific response of levels of TC/HDLC in one individual to dietary changes in PUFA may not follow

the relationship shown in this paper, particularly over a short period of time. Reasonable intake of PUFA over a period of several months is small compared with the large reserves of fatty acids in adipose tissue of most people.

Many physicians believe that drug therapy is needed because dietary changes alone are not sufficient to drastically reduce TC/HDLC. These views are supported by the American Heart Association, which asserted that diet can only lower cholesterol by up to 15%, and thus drug therapy may be necessary (32). This belief is derived from the fact that conventional treatment diets have relatively small amounts of EFA (often below 10 g/d), not enough to significantly change PUFA levels. Gronn *et al.* (19) used 36 g/d of n-6 and 6 g/d of n-3 derivatives to achieve minor reductions in TC/HDLC and total triglycerides over a 4-wk period. Substantial changes in body fat composition may take years to accomplish by dietary change. Given these factors and the random variability in lipid and fatty acid measurements, individual reductions in abnormal lipids may not reflect average statistical results, and perseverance is required for dietary changes to affect lipids.

This study shows that an inexpensive diet high in PUFA can drastically reduce TC/HDLC. Furthermore, eating PUFA to bring plasma levels closer to those of a healthy population may improve life expectancy through mechanisms other than their effect on TC/HDLC. Siscovick *et al.* (33) showed that reduced levels of n-3 PUFA in red blood cells are associated with increased risk of cardiac arrest. Patients who have low HDL and very high TC/HDLC (usually greater than 7), however, probably due to a genetic defect in making HDL rather than acquired factors (i.e., obesity or low PUFA), may not respond as expected to dietary changes in PUFA.

Treatment of cardiovascular disease through nutrition is less expensive than using drugs or surgery, and it can be equally or more effective. Diagnosis of lipid abnormalities through fatty acid analysis may lead to a new classification of dyslipidemia based on biochemical processes. Treatment of lipid abnormalities through manipulation of PUFA levels may change preventive medicine approaches and reduce health care costs. More research is needed to explore the relationship between lipids, *cis*, and *trans* fatty acids, and biochemical or genetic abnormalities associated with low HDL.

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Control of Endothelial Leukocyte Adhesion Molecules by Fatty Acids[1]

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ABSTRACT: Dietary balance of long-chain fatty acids (FA) may influence human susceptibility to pathological processes which involve the interaction of leukocytes with vascular endothelium, such as atherogenesis and inflammation. Such interaction is largely mediated by the *de novo* or increased expression of endothelial leukocyte adhesion molecules on vascular endothelial cells, able to tether and stably bind leukocytes onto the vessel wall, and by the production of leukocyte chemoattractants. Endothelial cells do not normally support high levels of leukocyte adhesion. They do so, however, when exposed to a number of stimuli, such as oxidized low density lipoprotein bacterial lipopolysaccharides, and inflammatory cytokines, which induce phenotypic changes generally referred to as "endothelial activation." We compared various FA in their ability to modulate endothelial activation by cytokines. FA included linoleic, arachidonic, oleic, eicosapentaenoic and, docosahexaenoic acid (DHA) as representatives of the n-6, n-3 polyunsaturated FA and of the monounsaturated FA. The n-3 FA DHA, and, to a lesser extent, oleate, at nutritionally compatible concentrations, were able to reduce endothelial expression of Vascular Cell and Adhesion Molecule-1 (VCAM-1). In further studies, DHA dose- and time-dependently reduced also the expression of E-selectin, Intercellular Adhesion Molecule-1, interleukin (IL)-6 and IL-8, in response to IL-1, IL-4, tumor-necrosis factor, or bacterial endotoxin. The magnitude of this effect paralleled its incorporation into cellular phospholipids. Also, coordinate with reduced surface adhesion molecule expression, DHA reduced the adhesion of human monocytes and of monocytic U937 cells to cytokine-stimulated endothelial cells. These effects were accompanied by a quantitatively consistent reduction in VCAM-1 mRNA, indicating a pretranslational control of adhesion molecule gene expression. These novel properties of FA as modulators of endothelial activation may help to explain the influence of dietary FA intake on atherogenesis and inflammation.

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Dietary lipid quantity and source can affect a variety of physiological responses of the human organism, thereby modulating the risk of development of diseases (1–4). In particular, dietary long-chain unsaturated fatty acids (FA), which are, quantitatively, minor lipid components in the diet, have been shown to modulate a wide number of cell responses to environmental stimuli (5–7). Among polyunsaturated fatty acids, a clear distinction of properties has emerged in these last years between the n-3 and the n-6 families. With some notable exceptions (8), epidemiological studies have suggested an inverse relationship between intake of n-3 (FA) and the incidence of cardiovascular disease (9–17), which is at least partially attributable to a reduced incidence of atherosclerosis (18). Consumption of n-3 FA diminishes atherogenesis in the nonhuman primate (19), as well as in other animal species (reviewed in Refs. 6 and 7). In addition to atherothrombotic diseases, inflammatory/immune diseases such as psoriasis, bronchial asthma, and thyrotoxicosis have been inversely associated epidemiologically with dietary intake of n-3 FA (9).

Efforts to understand the mechanism of action of n-3 FA have largely focused upon their effects on eicosanoid production. However, the hypothesis that n-3 FA act through alterations of eicosanoids cannot explain many facets of their biological activity, including the well-appreciated hypotriglyceridemic (20) and antihypertensive (21) effects. In addition, an ever growing number of other properties, potentially linked to the inhibition of atherogenesis or inflammation, appear to be also eicosanoid-independent. These include the decreased production of tissue factor (22) and cytokines (23) from monocyte–macrophages, the decrease in production of platelet-derived growth factor-like mitogens from endothelial cells (24) and monocyte–macrophages (25,26), and effects on the activation of immediate-early response genes (27). Thus, there is a general need to investigate new mechanistic explanations for anti-atherogenic and antiinflammatory effects of n-3 FA (28).

Atheroma formation and inflammation both require the adhesion of circulating leukocytes to the endothelium and their subsequent transendothelial migration (29–36). These processes depend on the endothelial expression of specific endothelial leukocyte adhesion molecules, such as Vascular Cell Adhesion Molecule-1 (VCAM-1), Intercellular Adhesion Molecule-1 (ICAM-1), and E-selectin, that recognize cognate ligands on leukocytes (37-39), and on the production of endothelial leukocyte-specific chemoattactants, such as Mono-

[1]A substantial part of the data presented in this paper has been previously published [*Arterioscler. Thromb. 14,* 1829–1836 (1994)], and are here reported with permission of the American Heart Association.

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Abbreviations: DHA, docosahexaenoic acid; EIA, enzyme immunoassay; EPA, eicosapentaenoic acid; FA, fatty acids; HSVEC, human saphenous vein endothelial cells; ICAM-1, Intercellular Adhesion Molecule-1; IL, interleukin; LPS, lipopolysaccharide; MHC-I, Major Histocompatibility Complex–Class I; TNF, tumor necrosis factor; VCAM-1, Vascular Cell Adhesion Molecule-1.

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cyte Chemoattractant Protein-1 (40), and interleukin (IL)-8 (41)

We hypothesized that n-3 FA may modulate endothelium-dependent mechanisms in atherogenesis and inflammation, such as the expression of endothelial leukocyte adhesion molecules and the production of endothelial chemoattractants. In more general terms, we hypothesized that endothelial cell responses to cytokine-stimulation (endothelial activation) could be modulated by changes in the relative proportions of specific FA in cell lipids.

METHODS AND RESULTS

Cultured human adult saphenous vein endothelial cells (HSVEC) were grown from unused portions of saphenous veins from coronary bypass surgery, as described previously. Cells were incubated for hours to days in 96-well plates with various unsaturated FA, including oleic acid (18:1, n-9), linoleic acid (18:2, n-6), arachidonic acid (20:4, n-6), eicosapentaenoic acid (EPA; 20:5, n-3) and docosahexaenoic acid (DHA; 22:6, n-3), the most abundant unsaturated FA, esterified in cell membrane phospholipids, used as sodium salts (Nu-Chek-Prep, Elysian, MN), and dissolved in Medium 199 containing 5% (volume to volume) fetal calf serum plus Endothelial Cell Growth Factor, heparin, l-glutamine, and antibiotics [Because fatty acids were dissolved in serum-containing medium (5% volume to volume), a 1 mM concentration of fatty acids corresponds, assuming a concentration of albumin in serum of about 4 g/dl, and a molecular weight for albumin of about 60,000, to about 0.5 mmol fatty acid/g albumin, and to a molar ratio of fatty acids to albumin of about 30 (30 mol fatty acids per mol of albumin.)]. Cells were then stimulated by cytokines [IL-1, tumor necrosis factor (TNF), IL-4] or bacterial lipopolysaccharide (LPS), still in the presence of added FA or in control conditions. The expression of cell-associated surface molecules was assessed by cell-surface enzyme immunoassays. These were carried out by incubating monolayers first with saturating concentrations of specific mouse monoclonal antibodies to human VCAM-1 (Ab E1/6), E-selectin (Ab H18/7), ICAM-1 (Ab HU 5/3), Major Histocompatibility Complex-Class I (MHC-I Ab W6/32)(43), or the monoclonal antibody E1/1, recognizing a constitutive and noncytokine inducible endothelial cell antigen (44); then with biotinylated goat antimouse IgG (Vector); and finally with streptavidin-alkaline phosphatase (Zymed). Repeated washings were performed between each incubation step. The surface expression of each protein was quantified by colorimetric reading of the optical density of the wells 15–60 min after the addition of a chromogenic substrate (*para*-nitrophenylphosphate), as described (43). Preincubation of HSVEC for ≥ 24 h with DHA and, to a lesser extent, with oleic acid, but not of other FA (including, notably, EPA), significantly decreased the expression of VCAM-1 induced by IL-1α or TNF, as assayed by cell surface enzyme-immunoassay (EIA). Therefore, further experiments focused on DHA DHA inhibited cytokine-induced expression of all three adhesion molecules assayed (VCAM-1 > E-selectin > ICAM-1) in a concentration-dependent manner (half maximal inhibition at <10 μM), at all time points examined during cytokine stimulation (Table 1). DHA inhibited VCAM-1 expression with all stimuli tested, including IL-1α (1–10 ng/mL), IL-1ß (1–10 ng/mL), TNFα (1–10 μg/mL), IL-4 (50–100 ng/ml), or LPS (1–10 ng/mL, from *Escherchia coli*).

TABLE 1
Endothelial Cell Expression of VCAM-1 and E-Selectin (o.d. mU) in Response to Interleukin-1 (10 ng/mL), in the Absence and in the Presence of DHA (10 μM), as a Function of Time After Cytokine Stimulation[a]

	VCAM-1		E-Selectin	
Time (h)	Control	DHA	Control	DHA
0	4 ± 5	5 ± 5	1 ± 2	1 ± 2
2	15 ± 3	10 ± 3	396 ± 20	205 ± 15
6	302 ± 12	140 ± 10	770 ± 42	402 ± 36
22	296 ± 25	100 ± 10	435 ± 50	230 ± 15

[a]Data (mean ± SEM) are pooled from two experiments, yielding >16 replicates for each time point. VCAM-1, Vascular Cell Adhesion Molecule-1; DHA, docosahexaenoic acid.

Flow cytometric analysis of suspended HSVEC confirmed decreased cytokine-stimulated cell surface expression of VCAM-1 by DHA. HSVEC were preincubated with medium supplemented with DHA (10 μM, 48 h) or in control conditions, after which IL-1α (10 ng/mL) was added for another 24 h. The surface expression of VCAM-1 was assessed incubating HSVEC, suspended in Hank's buffered saline solution with 3 mM EDTA, with the mouse anti-human monoclonal antibody E1/6 (specific for VCAM-1) and, subsequently, with goat anti-mouse $F(ab')_2$ form IgG (H + L), labeled with fluoresceine-isothiocyanate (Caltag), at 4°C. After washings and fixation in 1% formaldehyde, cell suspension was passed through a Becton-Dickinson (Rutherford, NJ) FACScan analyzer. DHA pretreatment significantly ($P < 0.01$) decreased the median intensity of fluorescence and splayed the distribution (Fig. 1), indicating that DHA limited cytokine-induced VCAM-1 expression in a substantial population of cells.

Various control experiments examined the selectivity of DHA actions on endothelial cells. Cell number (counting), morphology, and viability (Trypan blue exclusion) were not affected by preincubation with DHA concentrations up to 25 μM for periods up to 96 h. Total and cell-associated protein synthesis was assessed in the presence or absence of DHA. To this purpose, HSVEC were cultured in 12-well plates in the presence/absence of DHA, at 10 or 25 μM for 24–96 h, and subsequently stimulated with IL-1α for periods up to 24 h. At the time of IL-1 addition, ^{3}H-leucine (New England Nuclear, Boston, MA) was also added at 0.2 μCi/10^4 cells), and total protein synthesis was then evaluated by counting radioactivity after cold trichloroacetic acid precipitation of supernates and of total cell extracts at various time points, as described previously (45). DHA did not alter total and secreted protein synthesis, measured as incorporation of radioactivity into trichloroacetic acid-precipitable material after

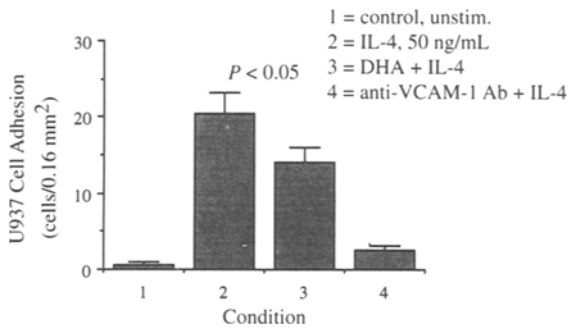


FIG. 1. Flow cytometric analysis of the effect of docosahexaenoic acid (DHA) on interleukin (IL)-1–induced Vascular Cell Adhesion Molecule-1 (VCAM-1) expression by endothelial cells. Human saphenous vein endothelial cells (HSVEC) were preincubated with medium supplemented with DHA (10 μM, 48 h) or in control conditions, after which IL-1α (10 ng/mL) was added for another 24 h. The surface expression of VCAM-1 was assessed detecting the binding of the mouse anti-human monoclonal antibody E1/6 (specific for VCAM-1). As controls, primary incubations were performed with a monoclonal antibody (E1/1) recognizing an endothelial surface antigen not induced by cytokines, or with a nonbinding antibody. The graph shows the mean ± SEM of the cell distribution of fluorescence intensity in the various conditions, as derived from original plots representing the intensity of fluorescence vs. cell number (total cells counted: 10^4 for each condition). Note the increase in fluorescence intensity after IL-1 stimulation, and its significant inhibition ($P < 0.01$, at Kolgomorov-Smirnov statistics) and spreading by DHA. Baseline fluorescence in unstimulated conditions is superimposable to that obtained with a nonbinding antibody, indicating the virtual absence of baseline expression of VCAM-1 in HSVEC; fluorescence after incubation of cells with an antibody (E1/1) directed against a constitutive endothelial antigen showed increase in mean fluorescence intensity compared to that obtained with a nonbinding antibody, and not modified by either cytokines or DHA (data not shown), indicating the presence of a noncytokine-inducible antigen on cell surface. Unstim, unstimulated.

labelling of the cells with ^{3}H-leucine. Nor did DHA alter the expression of certain other constitutive endothelial surface molecules, such as MHC-I and the antigen recognized by the monoclonal antibody E1/1 in EIA and flow-cytometry. These results indicate that the observed inhibition of cytokine-induced molecule expression by DHA was not due to a nonspecific toxic effect.

Inhibition of VCAM-1 expression by DHA depended on the time of incubation of HSVEC with DHA, requiring pretreatment and increasing for up to three days. The relationship of these kinetics with the reequilibration of polyunsaturated FA pools inside the cells was explored by parallel assessment of the FA composition of endothelial cells upon incubation with DHA. Relative amounts of DHA and of the individual chemical species of the n-3 and n-6 families were obtained by integration of peaks obtained by gas–liquid chromatographic separation of fatty acids from HSVEC monolayers, identified by comparison with retention times of FA standards. HSVEC, at passage 3–4, contained 5.7% of DHA, as percent of total FA, at baseline. Incubation of HSVEC with 10 μM DHA progressively increased total DHA incorporated in cellular lipids up to a maximum of 14.3% at 48 h, accompanied by increased total n-3 FA and a corresponding decrease of total n-6 FA. Such changes closely mimic those occurring in humans during dietary supplementation with n-3 FA, which produces a sustained enrichment of DHA in cellular phospholipids (46). The kinetics of DHA's effect on adhesion molecule expression roughly paralleled its incorporation into cellular lipids (Fig. 2).

In response to IL-1, TNF, or LPS, endothelial cells secrete IL-6 and IL-8, local mediators of the inflammatory response (41,47). DHA pretreatment also reduced HSVEC IL-6 and IL-8 secretion induced by IL-1, as measured by EIA in the supernate of HSVEC cultures, by commercially available kits (Genzyme, Boston, MA, and R&D, Minneapolis, MN) (Table 2). This is evidence that a vast number of changes in endothelial phenotype occurring during cytokine activation can be affected by specific FA.

Further experiments explored possible mechanisms by which DHA inhibits the endothelial expression of cytokine-induced molecules, using VCAM-1 expression as an index. The possibility that DHA acts by retroconversion to EPA, the direct precursor of eicosanoids of the n-3 series, seems highly unlikely. Despite some retroconversion of DHA to EPA in DHA-treated HSVEC (upon incubation with 10 μM DHA, EPA content increased from undetectable levels up to 2.1% at 72 h), exogenous EPA did not limit cytokine-induced VCAM-1 expression. In addition, treatment of HSVEC with the cycloxygenase inhibitors aspirin (100 μM) or indomethacin (5 μM), concentrations that completely suppressed IL-1–induced production of prostacyclin [assessed by EIA of its stable hydrolytic product 6-keto-PGF1α (Cayman Chemical,

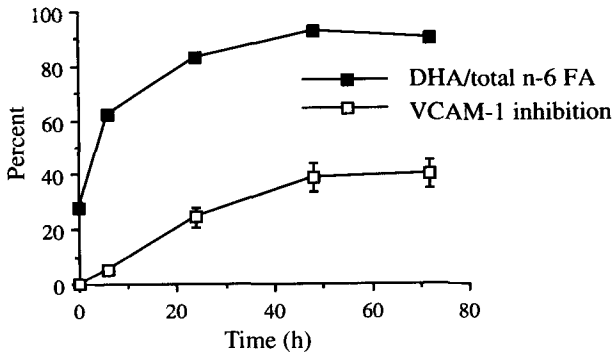


FIG. 2. The percent inhibition of IL-1α–stimulated VCAM-1 expression (open squares) as a function of DHA preincubation time, as obtained in cell-surface enzyme immunoassay experiments; each time point is the mean ± SEM from ≥3 experiments, each consisting of ≥16 comparisons between DHA-treated and control cell monolayers. The panel also shows the corresponding time course of DHA incorporation into total cell lipids (filled squares) from parallel HSVEC cultures, expressed as the percent ratio of DHA to total n-6 fatty acid (FA) in total cellular lipid, as determined by gas–liquid chromatography. There is a lag phase in the effect of DHA on VCAM-1 expression, with a minimal inhibition of VCAM-1 expression at a time (6 h), when the ratio DHA/total n-6 FA was half-maximal, after which there is a rough parallelism between incorporation and extent of VCAM-1 inhibition (reproduced with permission from the American Heart Association). Abbreviations as in Figure 1.

TABLE 2
The Effect of DHA (96-h preincubation) on the Secretion of IL-6 and IL-8 by Human Saphenous Vein Endothelial Cells[a]

	IL-6 (ng/10^6 cells)	IL-8 (ng/10^6 cells)
Control, no IL-1α	<0.2	0.5 ± 0.1
Control + IL-1α 10 ng/mL	9.0 ± 0.9	83.3 ± 3.1
DHA (10 μM) + IL-1α 10 ng/mL	6.5 ± 0.6[b]	69.3 ± 1.8[b]

[a]Values are mean ± SEM of protein concentrations assayed in duplicate in medium from ≥4 monolayers in each control unstimulated condition and ≥8 monolayers for each stimulated condition. Experimental conditions, in 12-well plates, are otherwise similar to those described for detection of cell surface antigens. Abbreviations as in Table 1.
[b]$P < 0.01$ in comparisons with corresponding control, stimulated condition (Student's *t*-test for unpaired data).

Ann Arbor, MI)] in the cell supernatants) did not alter DHA's effect (Fig. 3). Therefore, contrary to the traditional explanation for many effects of n-3 FA (48,49), DHA's actions on endothelial cells, which do not possess a 5-lipoxygenase pathway, does not appear to depend on conversion to eicosanoids. DHA also reduced levels of mRNA encoding VCAM-1 in IL-1α– or TNF-stimulated HSVEC (by 40 ± 10%, mean ± SEM, as determined by densitometry of Northern blots at 6–9 h in 4 different experiments), indicating that reduction of VCAM-1 synthesis after cytokine stimulation by DHA occurs, at least in part, at a pretranslational level (Fig. 4).

To study the functional consequences of DHA-induced reduction in the expression of adhesion molecules, we tested whether DHA treatment affected the adherence of human monocytic cells (U937 cells or flow-elutriated human monocytes) to HSVEC. U937 cells (American Type Culture Collection; NCAUR, Peoria, IL) were grown in RPMI medium 1640 containing 10% fetal calf serum and concentrated by centrifugation at 1×10^6 cells/mL. HSVEC were grown to confluency in the presence or absence of DHA (10 μM) in 6-well tissue culture plates for 96 h, after which human recombinant IL-4 (Genzyme) was added at 50 ng/mL for 24 h, in order to induce the expression of VCAM-1. For control, some monolayers were treated with a mouse anti-human monoclonal antibody (E1/6) against VCAM-1. The adhesion assay was performed by adding 1 mL of the concentrated U937 cell suspension to each monolayer under rotating conditions (63 rpm) at 21°C (50,51). After 10 min, nonadhering cells were removed by gentle washing, and monolayers fixed with 1% paraformaldehyde. The number of adherent cells was determined by counting six different fields using an ocular grid and a 20× objective (0.16 mm^2/field). Fields for counting adherent leukocytes were located at half-radius distance from the center of the monolayers. Adhesion of these leukocytes to endothelial cells under nonstatic conditions partially depends on VCAM-1 expression (50). Indeed, U-937 cell adhesion to HSVEC was practically absent without cytokine stimulation of HSVEC, and increased >30-fold after IL-4 stimulation (at 50 ng/mL) and >90-fold after IL-1 stimulation (10 ng/mL). This cytokine-stimulated adhesion was inhibited by the anti-VCAM-1 monoclonal antibody E1/6 (incubated for 1 h prior to the adhesion assay) by 40 ± 10% in the case of IL-1, and by 83 ± 12% in the case of IL-4, a more selective inducer of VCAM-1 in HSVEC, as in human umbilical vein endothelial cells (51). DHA pretreatment of HSVEC monolayers significantly inhibited IL-4–induced (Fig. 5) and IL-1–induced ad-

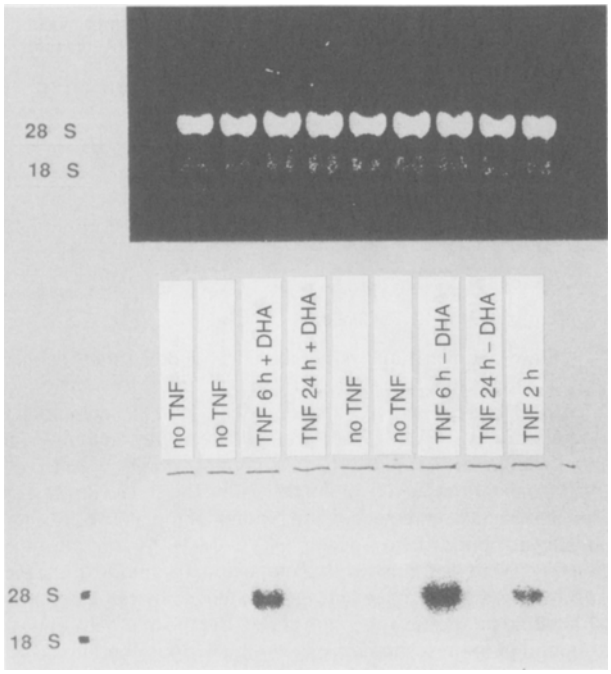


FIG. 4. Ethidium bromide staining for total RNA (upper panel), and Northern analysis of VCAM-1 mRNA (lower panel), at various time points after IL-1 stimulation, in DHA-treated HSVEC (10 μM, 48 h preincubation) and in control conditions. DHA preincubation decreases VCAM-1 mRNA (by about 50% by densitometry) during the time course of mRNA induction. Notice that the data on tumor necrosis factor (TNF) effect on VCAM-1 expression at 24 h has no match, for the design of the original experiment, in a lane with DHA + TNF for 24 h. Abbreviations as in Figure 1.

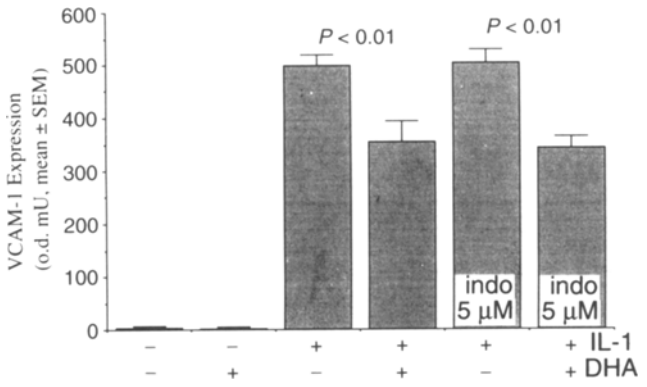


FIG 3. Indomethacin (indo, 5 μM), at a concentration able to suppress completely prostacyclin production by endothelial cells (data not shown), does not alter the effect of DHA (10 μM, 48 h preincubation) on IL-1-stimulated (24 h) VCAM-1 expression by endothelial cells. Similar results have been obtained with aspirin 100 μM. Abbreviations as in Figure 1.

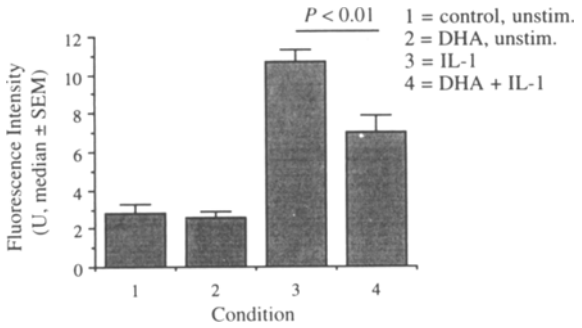


FIG. 5. The effect of DHA treatment of HSVEC on the adhesion of monocyte-like cells to HSVEC after cytokine stimulation. U937 cells adhere only to a minor extent to HSVEC not exposed to cytokines. The adhesion is dramatically increased by treatment of the monolayer with IL-4, a phenomenon which is almost entirely accounted for by the expression of VCAM-1, as it is almost completely inhibited by a treatment of the monolayer with saturating concentrations of the antibody E1/6 for 1 h prior to the assay. DHA pretreatment of the monolayer also inhibits the adhesion of U937 cells to a substantial extent, paralleling the inhibition of VCAM-1 expression as assessed by cell-surface enzyme immunoassay and flow-cytometry. IL-1–induced U937 cell adhesion was also reduced (10–40% in three different experiments, data not shown). Quantitatively similar results were obtained with flow-elutriated human monocytes. Bars show (mean ± SEM) cell counts/0.16 mm^2. Abbreviations as in Figure 1.

hesion, to a degree comparable to the inhibition of VCAM-1 surface expression, as assessed by direct assays of the protein. Similar results were obtained with flow-elutriated human monocytes (data not shown).

DISCUSSION

We show here that specific FA may alter the endothelial expression of a variety of cytokine-induced leukocyte adhesion molecules and of secretable protein products implicated in leukocyte recruitment and the local amplification of inflammation. The concentrations of FA (specifically of DHA) required for this modulation lie well within the range of those achievable during *in vivo* administration (46), suggesting that these *in vitro* findings may have a relevance in explaining dietary effects of FA. Also, the time-requirement for these effects to occur agrees with previous observations that most biological effects of changes in the n-3/n-6 FA balance are not acute, but require incorporation into cellular phospholipids (6,7). At variance from other known effects of n-3 FA (48,49), the presently described modulation appears to be totally independent of DHA metabolism to cyclooxygenase products.

We presently report a previously unsuspected difference in biological properties of DHA vs. EPA. It is unclear why DHA, but not EPA, was effective in reducing cytokine-induced VCAM-1 expression. EPA did potentiate the effect of DHA at concentrations at which EPA alone was ineffective. Also, EPA reduced IL-8 production stimulated by IL-1, although to a lesser extent than DHA. Several recent reports have recently described properties of DHA not shared by EPA (52–58), including a preferential (DHA vs. EPA) release by phospholipase A_2 from phosphatidylethanolamine, where most DHA usually accumulates (59). ^{14}C DHA radiolabelling of HSVEC, followed by thin-layer chromatographic separation of the various phospholipid classes, confirmed a preferential accumulation of DHA in phosphatidyle-thanolamine. The structure–activity relationship for these modulatory effects still require further testing, however.

DHA may regulate the protein expression of cytokine-induced molecules at different levels. Interference with cytokine-receptor binding or the initial events of signal transduction seems unlikely, as DHA limited the effects of structurally unrelated agonists (different cytokines and LPS), which bind to different receptors and use different signal transduction pathways. The reduction in VCAM-1 mRNA, consistent with data from measurement of protein expression, indicates a pretranslational level of action. As transcriptional regulation is the only type of regulation for endothelial leukocyte adhesion molecules reported so far (60), a transcriptional regulation by DHA seems likely, but has yet to be proven.

A recent report indicates that some polyunsaturated FA can be selective, redox-sensitive regulators of VCAM-1 gene expression in human vascular endothelial cells (61). In this report, linoleic and arachidonic acid could induce VCAM-1 expression in the absence of stimulating cytokines (61). Whether such induction can also be manipulated by substituting n-3 to n-6 FA also remains to be ascertained.

Dietary supplementation with n-3 FA concentrates can increase plasma DHA concentration from the low micromolar range up to more than 100 μM (46). Our results show that even much lower concentrations, as those achievable in plasma by moderate fish intake (62), can reduce cytokine-induced endothelial "activation." Our results thus provide a novel mechanism by which quantitatively minor dietary lipid components may influence cell physiology and the pathogenesis of certain inflammatory diseases, including atherosclerosis.

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Fats for the Food Industry: Implications for Cholesterol-Lowering

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ABSTRACT: The special needs of the food industry may not always coincide with what is healthiest for consumers. People always demand optimal taste, flavor and texture; they expect shelf-stability, convenience and novelty. The edible oils and fat industry has been particularly vulnerable in this regard but has tried, often very successfully, to modify its products to take into account the customer's preferences and their health. The following review illustrates some potential strategies in the retail and commercial sectors, especially in baking, frying and food service areas. Given the large increase in the consumption of convenient, small portion meals, the fat content and composition of such meals and products will critically influence further outcomes in areas related to cardiovascular disease, cancer, obesity and diabetes.

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Edible fats which are suitable for certain food service and bakery purposes need to be solid or semi-solid at room temperature. Commonly used are animal fats such as tallow or vegetable fats and blends comprising palm oil, coconut oil and partially hydrogenated soybean oil. Their public health disadvantage lies in the high content of lauric, myristic, palmitic or trans fatty acids (TFA), each of which has been shown to raise the plasma low density lipoprotein (LDL) levels in people, especially those at high risk from hypercholesterolemia.

Interesterification. Another approach is to harden liquid oils through interesterification. The rearrangement of certain fatty acids within triglyceride molecules changes the physical characteristics. One of the perceived constraints to this strategy is the possibility that the process of interesterification may increase the likelihood of that fat raising plasma LDL concentrations more than the native oil since the structure of the triglyceride may influence cholesterol metabolism.

Since unsaturated fatty acids normally occupy the middle or *sn*-2 position of a triglyceride molecule, and saturated fatty acids one of the outside (*sn*-1 or *sn*-3) positions, it is possible that an exchange of positions during interesterification will alter the influence of the fat on cholesterol metabolism. The fatty acid at the *sn*-2 position is conserved within the monoglyceride during fat absorption, whereas the outside fatty acids, absorbed as free fatty acids, are partially replaced by endogenous fatty acids in reconstituted triglycerides.

In the following experiments, we have re-examined the effects of transesterification on plasma lipids by comparing two blends of identical fatty acid composition, one of which has been randomized. The fatty acids were derived from palm kernel, palm stearin, palmolein and sunflower oils so that interesterification would lead to the enrichment of the *sn*-2 position with the saturated fatty acids, lauric, myristic and palmitic acids (Tables 1 and 2).

TABLE 1
Fatty Acid Profiles of Test Oil Blends (%)

	Test margarines		
Fatty acid	High linoleic-moderate (*trans*)	High-palm blend	Interesterified high-palm blend
$C_{10:0}$ capric	0	0.6	0.7
$C_{12:0}$ lauric	0	15.6	16.3
$C_{14:0}$ myriitc	0.3	6.4	6.2
$C_{16:0}$ palmitic	12.1	23.2	23.5
$C_{18:0}$ stearic	5.7	10.2	9.7
$C_{20:0}$ arachidic	0.3	0.3	0.3
$C_{22.0}$ behenic	0.4	0.2	0.2
$C_{18:1}$ *cis*, olic	21.7	24.5	24.2
$c_{18:1}$ *trans*, oleic	13.0	0	0
$C_{18:2}$ linoleic	44.0	18.6	18.4
$C_{18:3}$ α-linolenic	2.4	0.1	0.1

TABLE 2
Positional Distribution of Fatty Acids in Triglycerides (%)

	High-palm blend	Interesterified high-palm blend		
Fatty Acid	*sn*-3	*sn*-1,3	*sn*-2	*sn*-1,3
$C_{12:0}$	13.6	16.6	11.3	18.8
$C_{14:0}$	6.0	6.6	6.3	6.2
$C_{16:0}$	8.7	30.5	24.7	22.9
$C_{18:0}$	12.4	9.1	11.3	8.9
$C_{20:0}$	0.1	0.4	0.2	0.3
$C_{22:0}$	0.1	0.2	0.2	0.3
$C_{18:1}$	34.1	19.7	25.3	23.7
$C_{18:2}$	23.3	16.3	18.4	18.4
$C_{18:3}$	0.1	0.1	0.2	0.1

Twenty-seven hypercholesterolemic men participated in a double-blind, cross-over trial of three margarines: (i) high linoleic acid moderate trans fatty acids, (ii) high palm (pre-

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Abbreviations: CHD, coronary heart disease; LDL, low density lipoprotein; PUFA, polyunsaturated fatty acids; TFA, *trans* fatty acid.

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TABLE 3
Plasma Lipid Concentration During Dietary Periods[a]

Lipid mmol/L	Baseline	High-palm blend	Interesterified high-palm blend	High linoleic moderate *trans* blend	Significance
Total cholestrol					
mean	5.97	6.34	6.64	5.83	$P < 0.002$ for linoleic vs. interesterified blend and high palm
± SD	0.89	1.05	1.00	0.98	
LDL cholesterol					
mean	4.3	4.43	4.54	4.02	$P < 0.001$ for linoleic vs. high-palm and interesterified
± SD	0.85	0.94	0.88	0.85	
HDL cholesterol					
mean	0.92	1.07	1.05	0.96	$P < 0.001$ for linoleic vs. high-palm and interesterified
± SD	0.21	0.27	0.24	0.23	

[a]LDL, low density lipoproteins; HDL, high density lipoproteins.

dominantly lauric, myristic, palmitic, oleic and linoleic acids), and (iii) an interesterified form of the high palm. Both high palm margarines led to similar LDL cholesterol levels (4.43 ± 0.94 and 4.54 ± 0.88 mmol/L, respectively) and were significantly higher than the high linoleic fat (4.02 ± 0.85 mmol/L, $P < 0.001$) (Table 3). Interesterification transferred substantial palmitic acid to the *sn*-2 position and this was reflected in plasma chylomicrons.

The study also confirms that high-palmitic fats give higher plasma cholesterol levels than those rich in linoleic acid, even when the latter contain moderate amounts of TFA. Although further studies and comparisons of different mixtures of fatty acids, especially those likely to be used for commercial purposes, are required, the available data favor the conclusion that interesterification per se does not adversely influence plasma lipid concentrations through the rearrangement of the triglycerides and provides a strategy for hardening fats which is an alternative to using TFA.

A recent report by Zock *et al.* (1) also showed that the positional distribution of palmitic acid in two contrasting margarines failed to affect plasma LDL or high density liproprotein cholesterol levels significantly.

Commercial frying oils. It is estimated that up to one third of fat energy is derived from commercially prepared foods with fried foods probably contributing a significant proportion of this. There is good evidence that this contribution is likely to rise with trends toward an increase in expenditure in snacks and takeaway foods in the last ten years.

A major use of cooking fats is in deep fat frying where it functions as heat transfer medium and contributes desirable flavor and texture to fried foods. Functionally, such oils need to be stable at the high temperature and moisture exposure,which is characteristic of commercial usage where oils are used repeatedly and frying may occur over several hours. During heating, polyunsaturated fatty acids (PUFA) polymerize, creating a viscous oil which produces a greasy product. Furthermore, as in the case of stored snack items, the stability of the fat will determine shelf-life, the more saturated the oil the more stable it is to oxidative and hydrolytic breakdown.

Major frying fats in Australia are tallow and palm oil, both of which are highly stable to oxidation and also have good palatability and mouth-feel. However, they are also highly saturated fats making them nutritionally undesirable in countries that experience high rates of coronary heart disease (CHD).

Linolenic rich oils such as canola and soybean oils are particularly susceptible to undesirable changes. Substantial hydrogenation of these oils renders them more stable, but results in levels which may negate their lipid lowering potential. Sunflower and safflower oils are also unsuitable commercial frying oils because of their high PUFA content. In contrast, oils high in monounsaturated fatty acids are relatively resistant to oxidation. Recently, a high oleic genetic variant of sunflower oil (Sunola™) has been developed which has the stability and potentially the nutritionally desirable characteristics required in a frying fat. Solid partially hydrogenated fats with moderate levels of TFA, but with adequate linoleic acid, have also been developed for baking and have been shown to have cholesterol lowering attributes (2).

We have compared the effects on plasma cholesterol levels of three types of cooking fats suitable for frying. Twenty-three hypercholesterolemic men and women participated in a double-blind, cross-over trial of palm oil, a high oleic genetic variant of sunflower oil (Sunola™) and a newly developed semisolid blend with moderate TFA (20%) but designed to have lipid lowering potential through unsaturates (55% *cis*-oleic and 9% linoleic). The test blends provided 20% energy. Palm oil resulted in the highest LDL concentration (4.18 ± 0.81 mmol/L), with Sunola (3.77 ± 0.58 mmol/L) and

TABLE 4
Mean Differences in Plasma Lipids (mmol/L ± SD) Between Test Diets

Plasma lipids	Palm-Sunola™	Palm-test	Sunola™-test
Total cholesterol	0.51 ± 0.37[a]	0.31 ± 0.32[a]	-0.21 ± 0.30[c]
LDL Cholesterol	0.42 ± 037[a]	0.28 ± 0.41[a]	-0.14 ± 0.37
Triglyceride	0.17 ± 0.39	-0.03 ± 0.39	-0.21 ± 0.40[c]
HDL cholesterol	0.04 ± 0.09[b]	0.08 ± 0.10[b]	0.04 ± 0.09[c]

[a]$P < 0.001$ palm vs. mean of Sunola™ and test.
[b]$P < 0.01$ palm vs. mean of Sunola™ and test.
[c]$P < 0.05$ Sunola™ vs. test. See Table 3 for abbreviations.

the experimental blend (3.88 ± 0.70 mmol/L) resulting in the lower levels. The difference between the latter two blends was statistically insignificant (Table 4).

The purpose of this study was to compare the effects on plasma cholesterol levels of three types of frying fats. Palm oil is widely used in commercial practice and monounsaturated oils are finding favor on health grounds. One disadvantage of the latter is its liquid state and for this reason, we tested a semi-solid formulation in which the mix of fatty acids was likely to be less cholesterol raising than palm oil.

The results show that compared to palm oil, Sunola™ oil led to significantly lower total and low-desnity lipoprotein. The test blend behaved similarly to Sunola™ in terms of LDL cholesterol lowering and this occurred despite the increase of $C_{18:1}$ *trans*. Palmitic acid is the major saturated fatty acid in the palm oil blend. An approximate 10% energy substitution of palmitic by oleic acid in our mildly hypercholesterolemic subjects resulted in a fall of 0.50 mmol/L in total cholesterol. The current weight of evidence suggests that palmitic acid is cholesterol-elevating relative to oleic acid and that this effect is particularly pronounced in populations where the majority of people have plasma total cholesterol concentrations above 5 mmol/L. Palmitic acid is the major saturated fatty acid in the Australian diet, contributing 48% of total saturated fatty acids or 7% energy. Snack foods, baked goods and confectionery currently contribute 20% of the total palmitic acid consumed. The replacement of palmitic acid, where possible in the food industry, is a desirable goal.

In the present study, *trans* intake was estimated to be less than 1% energy in the palm and Sunola™ phases primarily from dairy and beef products. During the test blend phase, *trans* intakes increased by 4.8% energy with a concomitant increase of 1.5% energy in linoleic acid. It has been suggested that it is the overall ratio of linoleic to palmitic acid in commercial oil blends that determines LDL lowering potential and that, if this is adequate, the inclusion of modest amounts of TFA will not negate this (2). It is therefore of interest that the ratios of linoleic/palmitic acid were similar in Sunola™ and the test blend at about 1:1 and that there was no significant difference in LDL cholesterol between the two oils.

In conclusion, monounsaturated oils such as Sunola™ are clearly preferable to palm oil in the food service industry, in terms of cardiovascular risk. Further, it is possible to design formulations of blends, such as the test blend used in this study, which are partially solid and suitable for commercial frying purposes but have a favorable effect on plasma LDL cholesterol levels compared with palm oil. While economically Sunola™ oil is more expensive than tallow or palm oil, its stability to continuous deep fat frying makes its overall cost reasonably competitive with current frying fats.

Biological manipulation of milk fat. Milk and butter intake has been clearly associated with higher CHD rates in different countries (3). Correlation with cardiovascular disease mortality is likely to be mediated by the effect of dairy fats on the plasma LDL cholesterol.

Dairy products (excluding butter) make a substantial contribution to saturated fat intake (25–29%) in Westernized countries. In Australia, fats from dairy foods currently comprise 25% of the total fat intake and as much as 40% of the saturated fatty acids. The hypercholesterolemic effect of dairy products has been conclusively demonstrated in numerous controlled studies. Two of the principal saturated fatty acids in butterfat, namely myristic and palmitic acids, have been identified as major dietary factors that raise LDL cholesterol.

In vivo fat-modified dairy products are an innovation involving a technology in which protected unsaturated lipids are fed to ruminants resulting in milk and tissue lipids with reduced saturated fatty acids. We examined the impact of these novel dairy fats on plasma lipids in a human dietary trial (Table 5). Thirty-three men and women participated in an eight week randomized cross-over trial comparing fat-modified and conventional dairy products. During the test periods, the fat-modified products resulted in a 0.28 mmol/L (4.3%) lowering of total cholesterol which was significant at $P < 0.001$. Most of this fall was in LDL cholesterol which fell by 0.24 mmol/L ($P < 0.001$) whereas high density lipoprotein

TABLE 5
Mean Fatty Acid Profiles of Control and Fat-Modified Dairy Products

Fatty acid	Control (%)	Fat-modified (%)
$C_{4:0}$ butyric	5.7	5.5
$C_{6:0}$ caproic	2.7	2.5
$C_{8:0}$ caprylic	2.9	1.3
$C_{10:0}$ capric	2.8	2.3
$C_{12:0}$ lauric	3.3	2.3
$C_{14:0}$ myristic	10.0	6.7
$C_{16:0}$ palmitic	25.9	15.5
$C_{18:0}$ stearic	11.7	14.3
$C_{18:1}$ oleic	22.8	35.3
$C_{18:2}$ linoleic	1.5	6.9
$C_{18:3}$ linolenic	0.7	2.2
Unidentified (incl. TFA)[a]	10.0	4.2

[a]TFA, trans fatty acid.

TABLE 6
Mean (± SD) Plasma Lipoproteins During Baseline, Control, and Fat-Modified Phases

Plasma lipids (mmol/L)	Baseline	Control	Fat-modified
Total cholesterol	5.89 ± 0.89	6.50 ± 0.98	6.22 ± 0.82[a]
LDL cholesterol	3.95 ± 0.75	4.49 ± 0.90	4.25 ± 0.71[a]
HDL cholesterol	1.19 ± 0.30	1.30 ± 0.33	1.28 ± 0.33
Triglycerides	1.67 ± 0.85	1.57 ± 0.72	1.54 ± 0.66

[a]$P < 0.001$ control vs. fat-modified. See Table 3 for abbreviations.

cholesterol and triglycerides remained essentially unchanged (Table 6).

The cows were fed supplements of a primarily monounsaturated source by using canola seed (protected from hydrogenation in the rumen) and the additional use of Vitamin E in the ration which is also transferred to the secreted milk to stabilize PUFA.

It can be concluded that modifying the fat composition of milk and related dairy products through the substitution of palmitic and myristic acids by oleic, linoleic and linolenic acids will result in a significant improvement in the plasma lipoprotein profile in humans. We observed a fall of 0.28 mmol/L (4.3%) in total plasma cholesterol by substituting the fat modified dairy products for conventional products. Cholesterol-lowering intervention trials suggest that this alone could reduce clinical coronary disease by 9% without appreciably changing eating habits.

TFA. TFA in the food supply have recently generated controversy which appears to many nutritionists to have gained undue notoriety. Scientific discussion should focus mainly on the link between TFA and coronary heart disease prevalence and risk. This is clearly the major current health issue. The least controversial issue relates to the effects of TFA on plasma lipids. While much of the data were derived from dietary interventions with partially hydrogenated fats and oils and only more recently with specified TFA, it is agreed that LDL cholesterol is raised. However, while the LDL are higher than with the corresponding unhydrogenated oils, the levels are marginally lower than occur with highly saturated fats such as butter or palm oil (4). Since this is clearly dose related, the contribution which TFA make to the total LDL raising potential of the diet will depend on the mix of foods chosen by an individual. Thus the effect of saturates in the diet will substantially exceed that of TFA, given their relative proportions in edible fat eaten by most people. One major recommendation would be supported by most objective nutritionists: to encourage the edible oil industry to introduce new technologies for hardening oils which will reduce the TFA load. However, this must not be interpreted, as has been occurring, that saturated fats as butter are preferable. The scientific evidence is clearly against that extrapolation.

Less certain are the effects of TFA on HDL cholesterol-lowering and on Lp(a) concentrations (4,5). It is likely that significant HDL lowering becomes important only at higher intakes of TFA such as are eaten only by a small minority. The problem inherent in Lp(a) measurement and the paucity of reports that Lp(a) are raised leave that issue in the uncertain basket (4).

Most controversial is the alleged link between TFA consumption and CHD. Of some concern is that in the two most influential studies, by Ascherio *et al.* (6) and Willett *et al.* (7) who ranked CHD risk by quintile of TFA intake, the lowest risk was in the mid-quintile and not in the lowest quintile. This would lead to the conclusion that eating around 3–4 g TFA daily is more protective against CHD than eating none at all.

Of further interest is that in some case-control studies (8) in which tissue TFA concentrations were the indices of TFA consumption, the link with CHD occurred with *trans* 16:1 which is derived from animal fat and not from hydrogenating vegetable oil. By contrast tissue *trans* 18:1, derived through hydrogenation, was not associated with CHD.

More recently, two reports do not confirm the American studies. In eight European countries and Israel, the TFA content of adipose tissue (a reliable index of the kind of fat eaten during the preceding year or so) was measured in case-control studies (671 men after myocardial infarction and 717 controls). There was no association between *trans* oleic acid (elaidic acid) and clinical events (9).

In a second study, from England, there was again no association between the *trans* isomers of oleic and linoleic acids and sudden cardiac death (10). Given the objective for further reductions in saturated fat intake, one would like to see TFA included in whatever reductions take place in total fat consumption.

Structured Lipids. These are families of triglycerides structured through interesterification to provide novel, designer fats not found in nature. They are generally mixtures of long, medium and short chain fatty acids, designed for specific health benefits and functional suitability for use in bakery products and confectionery, where fat substitutes may not perform well.

The health perspectives are to reduce energy content, lower plasma cholesterol and to improve body functions such as immunity. The principles have been to use poorly or less well absorbed fatty acids such as behenic or stearic acids and interesterifying them with medium chain e.g., octanoic or decanoic acids or short-chain e.g., butyric or propionic acids. Two such products, Caprenin and Salatrim, appear to have energy values of only five kal/g, but their potential to lower cholesterol levels is not established. Dairy fats have also been interesterified with medium chain fatty acids to achieve the above health benefits. Fish oil fatty acids have been interesterified with short and medium chain fatty acids to achieve better absorption of eicosapentaenoic acid while delivering more butyrate into the intestine to hopefully benefit the gut mucosa.

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Factors Affecting Resistance of Low Density Lipoproteins to Oxidation

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ABSTRACT: Oxidation resistance (OR) of low density lipoproteins (LDL) is frequently determined by the conjugated diene (CD) assay, in which isolated LDL is exposed to Cu^{2+} as prooxidant in the range of 1–10 μM. A brief review on major findings obtained with this assay will be given. A consistent observation is that vitamin E supplements or oleic acid–rich diets increase OR. Oxidation indices measured by the CD assay and effects of antioxidants very significantly depend on the Cu^{2+} concentration used for LDL oxidation. For medium and high Cu^{2+} concentrations, the relationship between lag time and propagation rate can be described by a simple hyperbolic saturation function, which has the same mathematical form as the Michaelis–Menten equation. At medium and high Cu^{2+} concentrations (0.5 to 5 μM), vitamin E increases lag time in a dose-dependent manner. The increase is higher for 0.5 μM Cu^{2+} as compared to 5 μM. At low Cu^{2+} concentrations (0.5 μM or less), the mechanism of LDL oxidation changes. Significant oxidation occurs in a preoxidation phase, which commences shortly after addition of Cu^{2+}. Preoxidation is not inhibited by vitamin E. It is concluded that much additional work is needed to validate the importance of oxidation indices derived from CD and similar assays.
Lipids 31, S-71–S-76 (1996).

Oxidative modification of low density lipoproteins (LDL) most likely plays an important role in the development of atherosclerosis (1,2), but many questions on factors determining oxidation resistance (OR) of LDL and how reliable indices characteristic for OR can be measured remain unanswered (3). At present, we have very limited knowledge on how and where oxidation of LDL is initiated *in vivo*, the knowledge of which would be crucial in identifying important factors for protection of LDL against oxidative modification *in vivo*. At present, it is generally believed that lipid peroxidation is the process most relevant for producing LDL with high atherogenic potential. Therefore numerous studies have aimed to evaluate the role of antioxidants and other factors for protecting LDL against lipid peroxidation. Examination of recent literature clearly shows that most laboratories working on this subject expose isolated LDL to prooxidants (e.g., copper ions, azo-initiators, ultraviolet light, cells) and measure progress and/or extent of lipid peroxidation. One such model to measure kinetic parameters that has become very popular is copper ion (Cu^{2+})–promoted oxidation in conjunction with continuous measurement of the conjugated diene absorption at 234 nm (CD assay). It should be emphasized that we have developed this CD assay primarily as a time marker (4) useful for basic kinetic studies, but we did not intend to propose the assay for routine clinical-chemical analyses of OR of LDL. It was perhaps the report by Regnström and colleagues (5) on the inverse relationship between OR of LDL (measured by lag time of Cu^{2+}-dependent oxidation) and severity of myocardial infarction that led to the assumption that the CD assay is a biologically relevant model and gives indices (e.g., lag time) of clinical importance. In the meantime, many studies were published in which this assay was used to explore the importance of antioxidants and dietary fats for OR of LDL and to evaluate relationships between OR and severity and progression of atherosclerosis in humans and animals. A selected list of studies

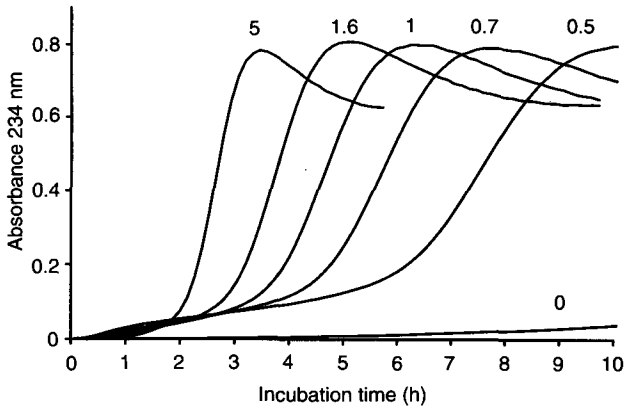


FIG. 1. Oxidation of LDL with medium to high Cu^{2+} concentrations. LDL (0.1 μM) in phosphate-buffered saline was oxidized at 30°C with the indicated concentrations (0.5 to 5.0 μM) of Cu^{2+}, and conjugated dione (CD)-curves were recorded. Also shown is CD increase without added Cu^{2+} (0).

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Abbreviations: CD, conjugated diene; LDL, low density lipoprotein; OR, oxidation resistance.

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TABLE 1
Selected Studies (1994/1995) on Oxidation Resistance of Low Density Lipoproteins (LDL) Measured by the Conjugated Diene Assay

Authors	Major finding
1994	
Stalenhoef *et al.* (6)	Patients with familiar defective apolipoprotein B had decreased *t*.[a]
Kontush *et al.* (7)	In 20 healthy subjects vitamin E content did not correlate with *t*.
Reaven *et al.* (8)	In combination with vitamin E, 18:1[b] increased the OR[c] and 18:2[d] decreased the OR of LDL subfractions.
Sasahara *et al.* (9)	In probucol-treated monkeys on cholesterol diets, the increase in *t* correlated with reduction in lesion size.
Nenseter *et al.* (10)	Cholesterol feeding reduced *t* of rabbit LDL; thiobarbituric acid reactive substances and peroxides were increased.
Fruebis *et al.* (11)	In probucol-treated rabbits (1% in diet) *t* increased from 2 to > 16 h.
Thomas *et al.* (12)	In primates with different fatty acid diets, *t* correlated with vitamin E in cases of polyunsaturated fatty acid diet, yet not with saturated and monounsaturated fatty acids.
Kleinveld *et al.* (13)	In Watanabe heritable hyperlipidemic rabbits, vitamin E treatment increased *t* and decreased *v*.[e]
Maggi *et al.* (14)	In 37 uremic patients, *t* decreased, *v* increased, and *t* correlated with vitamin E.
Tribble *et al.* (15)	OR of LDL subfractions was correlated with their ubiquinol-10 and vitamin E contents.
Tsai *et al.* (16)	Patients with poorly control insulin-dependent diabetes mellitus showed shorter *t*, but the distribution of LDL subfractions did not differ from control subjects. Uric acid and vitamin A decreased in diabetes.
Nyyssonen *et al.* (17)	Supplementation with vitamin E, β–carotene, ascorbate, and selenium increased *t* and decreased *v* of oxidation of LDL + very low density lipoproteins.
1995	
Winklhofer-Roob *et al.* (18)	LDL of cystic fibrosis patients had decreased *t*. Vitamin E and β–carotene supplementation normalized the OR; *t* correlated with vitamin E.
Jialal *et al.* (19)	Minimum dose of 400 IU/d vitamin E was needed to significantly increase the OR of LDL.
Suzukawa *et al.* (20)	Supplementation of low-dose vitamin E for 3 wk protected LDL against oxidative modification (increase in *t* and decrease in *v*) and decreased *v* in high-density lipoprotein.
Livrea *et al.* (21)	Vitamin A supplements increased *t* and decreased *v*.
Croft *et al.* (22)	In 45 healthy individuals, vitamin E was not correlated with *t*; *v* negatively correlated with 18:1 and positively with 18:2.
Gaziano *et al.* (23)	β–carotene supplementation did not increase *t*.
Mosinger (24)	*t* and CD_{max}[f] correlated with LDL cholesterol.

[a]*t* = Lag time.
[b]18:1 = Oleic acid.
[c]OR = Oxidation resistance.
[d]18:2 = Linoleic acid.
[e]*v* = Propagation rate.
fCD_{max} = Extent of oxidation.

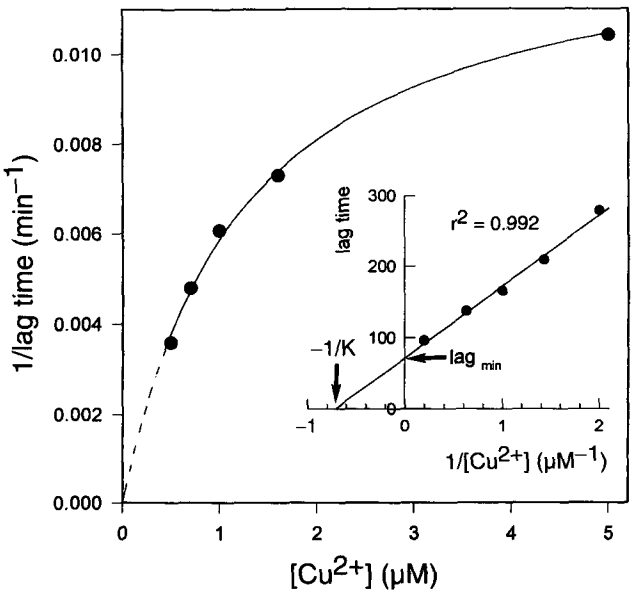


FIG. 2. Relationship between lag time and the Cu^{2+} concentration. Data derived from Figure 1. Lag time is the intercept of the tangent to the slow and fast increase of conjugated diene (4).

published recently (1994 to March 1995) can be found in Table 1.

Interpretation of such studies is usually confused by the fact that quite different assay conditions are used. In retrospect, we feel that it would have been important to emphasize that oxidation indices derived from a CD curve strongly depend on concentration of Cu^{2+}, LDL, temperature, pH value and other extrinsic factors. We have shown (25) that the temperature dependence of lag time and propagation rate can fully be described by the Arrhenius law; core melting of LDL has no or only a minor effect on OR.

In this study, we performed a systematic investigation to elucidate the effect of Cu^{2+} concentration on OR of LDL. We show for the first time that the copper concentration has not only a pronounced effect on the mechanism of LDL oxidation but also on the antioxidant action of vitamin E.

MATERIAL AND METHODS

Human LDL was prepared by ultracentrifugation using a single-step discontinuous gradient in a Beckman (Palo Alto, CA) NVT 65 rotor (26). LDL oxidation was carried out as previously described (27) with 0.1 μM LDL (equal to 0.25 mg total LDL mass/mL) in phosphate-buffered saline pH 7.4 and $CuSO_4$ as prooxidant. Cu^{2+} concentrations are given in the text. CD curves were recorded in a Beckman DU-640 spectrometer equipped with an auto-cellholder with six cuvettes and a Peltier-element temperature control. In all experiments, the temperature was 30°C (27). LDL antioxidants were determined in principle as described in (2). Loading of LDL with α-tocopherol was performed as described previously (28).

RESULTS AND DISCUSSION

Oxidation of LDL with medium and high Cu^{2+} concentrations. The original protocol for measurement of CD curves recommended 0.1 μM LDL (equal to 0.25 mg LDL mass/mL) and 1.6 μM Cu^{2+} (4). This gives lag times of about 30 to 180 min depending on the donor (2). Figure 1 clearly shows that increasing Cu^{2+} reduces lag time and increases rate of propagation, whereas lowering Cu^{2+} from 1.6 to 0.5 μM results in increase in lag time and decrease in propagation rate. The extent of oxidation, characterized by the CD_{max} value prior to onset of decomposition, is virtually independent of Cu^{2+} concentration. Figure 2 shows that for Cu^{2+} concentrations ≥ 0.5 μM the relationship between lag time and Cu^{2+} can be described by Equation 1, where lag is the lag time in minutes obtained with a certain concentration (μM) of added $[Cu^{2+}]$; lag_{min} is the minimum value for the lag time; and K is the Cu^{2+} concentration for lag = 2 lag_{min}:

$$\frac{1}{\text{lag}} = \frac{1}{\text{lag}_{min}} \cdot \frac{[Cu^{2+}]}{K + [Cu^{2+}]}$$

Figure 3 shows that propagation rate v approaches a maximum with increasing Cu^{2+} concentration. This relationship can be described by Equation 2 (25), where v is the propagation rate in ΔA_{234}/min or μM dienes/min obtained from the first derivatives of the CD curve; v_{max} is the maximum value for the propagation rate; and K′ is the Cu^{2+} concentration for $v = v_{max}/2$:

$$v = v_{max} \cdot \frac{[Cu^{2+}]}{K' + [Cu^{2+}]}$$

Note that both Equations 1 and 2 represent hyperbolic saturation functions, with the same mathematical form as the Michaelis–Menten equation. In analogy to Line-Weaver-Burk plots, strict linear relationships for the lag time or propagation rate can be obtained by plotting lag vs. the reciprocal value of $[Cu^{2+}]$(Fig. 2 inset) or by plotting $1/v$ vs. the reciprocal value of Cu^{2+} (Fig. 3 inset).

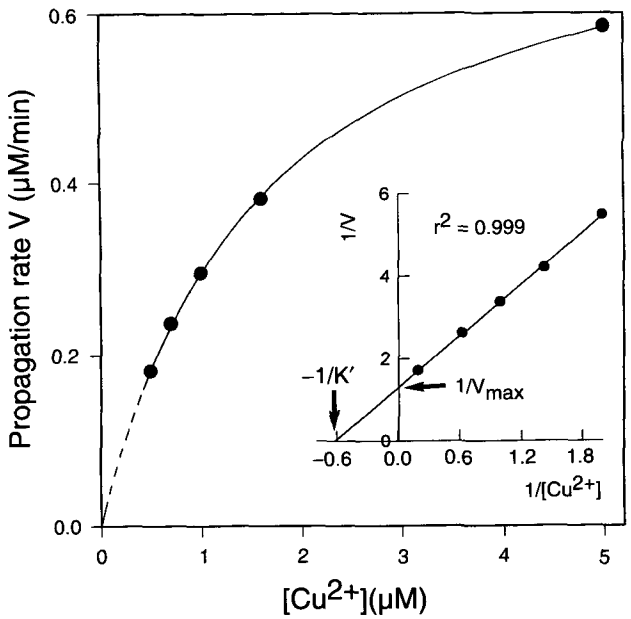


FIG. 3. Relationship between rate of propagation (v) and Cu^{2+} concentration. Data derived from Figure 1, v was determined from the first derivative of the conjugated diene curves (27).

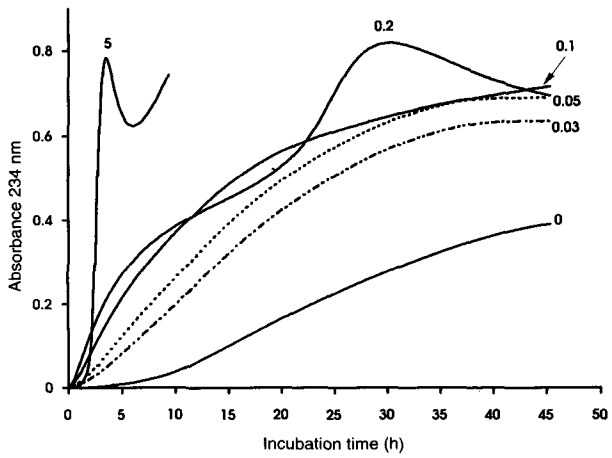


FIG. 4. Oxidation of low density lipoprotein (LDL) with low Cu^{2+} concentration. The same LDL sample as was used in Figure 1 was oxidized with 0.2, 0.1, 0.05, and 0.03 μM Cu^{2+}, and the conjugated diene increase was measured. Also shown for comparison is oxidation with 5 μM Cu^{2+} and oxidation without added Cu^{2+}.

Oxidations with low Cu^{2+} concentrations. In previous LDL oxidation studies performed and published by us (2,29) and other authors (Table 1) Cu^{2+} concentrations of 0.5 μM or higher were used. As shown in Figure 1, such concentrations are capable to fully oxidize LDL within a period of 10 h or less. The reason for using $Cu^{2+} > 0.5$ μM was simply a logistic one: such conditions allow us to oxidize LDL within several h, a reasonable time to complete experiments within a working day or during an overnight run. Moreover, CD curves measured under such conditions show the classical profile with a lag phase, propagation phase, and decomposition phase. We had no rational reason to presume that this trend to longer lag times and lower propagation rates would not continue if Cu^{2+} is further decreased. When the same LDL sample used for the experiments shown in Figure 1 was oxidized with Cu^{2+} of 0.2 μM or less, however, the shapes of the CD curves unexpectedly changed completely (Fig. 4).

In the experiment with 0.2 μM Cu^{2+}, oxidation occurred in several consecutive steps. The first steps, denoted by "preoxidations," commenced a few min after addition of Cu^{2+}, lasted about 10 h, and caused heavy oxidation of LDL with about 50% of CD_{max}. Then followed an inhibited interval with slow increase of CD and a second rapid oxidation leading to CD_{max}. The inhibited interval and the second rapid rise of CD most likely represent the classical lag phase and propagation phase characteristic for oxidation with medium or high Cu^{2+} concentrations. With Cu^{2+} concentrations of 0.1, 0.05, and 0.03 μM, CD curves exhibited the preoxidation stage and the inhibited interval, but no propagation phase. Preoxidation rate decreased with decreasing Cu^{2+} concentration (0.5 to 0.03

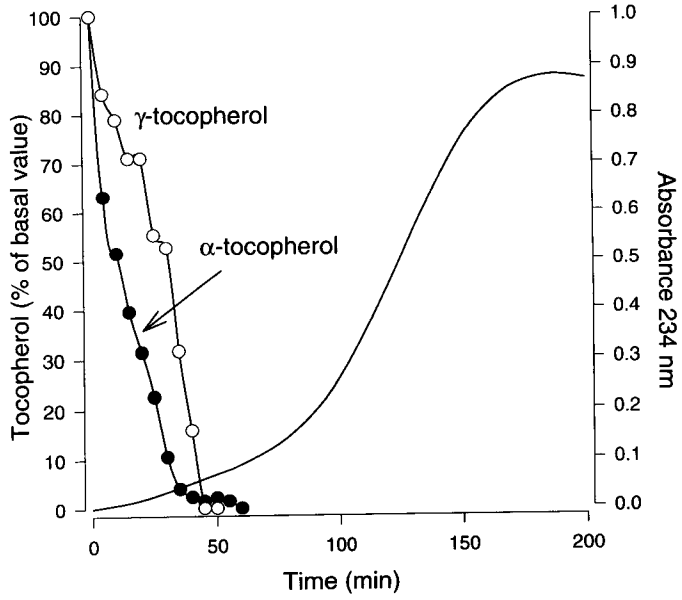


FIG. 5. Temporal relationship between loss of vitamin E and oxidation (conjugated diene-increase) of low density lipoprotein (LDL) with 1.6 μM Cu^{2+}. Initial concentrations were 0.609 μM α-tocopherol, 0.038 μM γ-tocopherol, and 0.1 μM LDL.

μM), but was in all instances higher than the rate during the lag phase in oxidations with 0.5 to 5.5 μM Cu^{2+}. Figure 4 also shows that the LDL sample without added Cu^{2+} was heavily oxidized after 45 h.

Antioxidants delay onset of propagation. It has previously been shown (2,29) that onset of propagation is preceded by the lag phase during which the LDL becomes progressively depleted of its antioxidants, with α-tocopherol as the first and β–carotene as the last to be depleted. This chronology was observed in LDL oxidations with medium and high Cu^{2+} concentrations in the range of about 1 to 10 μM. We have repeated these experiments, and results were in full agreement with our previous observations. A representative example for loss of vitamin E is shown in Figure 5. It has also been reported by us (2,29) and other authors (Table 1) that loading LDL with α-tocopherol either *in vitro* or by vitamin E supplements increases the length of the lag phase and therefore delays onset of propagation. As shown in Figure 6, the effect of α-tocopherol on lag time strongly depends on the concentration of Cu^{2+} used for oxidation. The effect is much more pronounced in oxidations with 0.5 μM Cu^{2+}, as compared to 5 μM Cu^{2+}. This may explain why some authors using high Cu^{2+} concentrations found no effect of vitamin E on lag time.

Antioxidants cannot prevent "preoxidation." Figure 7 shows an oxidation of LDL with 0.2 μM Cu^{2+}. The CD curve clearly exhibits a step wise oxidation with a preoxidation phase lasting about 10 h. During this stage, CD reached about 50% of CD_{max}, indicating that LDL becomes heavily oxidized prior to the onset of the propagation phase, which occurred at about 24 h. The average rate of diene formation during the first 10 h was 21 nM/min. We also measured the consumption of antioxidants and found that their concentration only slowly decreased during the preoxidation phase. After 10 h, a time-point when LDL was already heavily oxidized, only 38 and 12% of α- and γ-tocopherol were consumed (Fig. 7). Consumption of β–carotene and lycopene was 55 and 75%, respectively (data not shown). Average consumption rate of α-tocopherol (initial content 3.2 mol/mol LDL) was 0.2 nM/min, 100 times less than the CD production rate. From these experiments and others performed under comparable conditions, it is evident that LDL antioxidants do not prevent preoxidation promoted by low Cu^{2+} concentrations. In one experiment (data not shown), LDL was loaded *in vitro* with α-tocopherol and then subjected to oxidation with a low Cu^{2+} concentration. α-Tocopherol had no effect on the rate of CD increase during the preoxidation phase, but the length of this phase was prolonged. At present, it is not clear whether the preoxidation reflects perhaps tocopherol-mediated propagation (30) or is due to other, specific elementary reactions characteristic for low rate of initiation.

At present, we have very limited knowledge on how and where LDL oxidation is initiated *in vivo* and how quickly oxidation proceeds (1,2). This information is, however, crucial to intelligent design of assays, which give biologically relevant answers on factors determining OR of LDL *in vivo*. Many studies based on the classical CD assay (2, 29, and references in Table 1) revealed that vitamin E supplements or an oleic acid–rich diet significantly increase OR of LDL. Case-control studies and intervention trials with antioxidants (Table 1) also suggest some relationships between OR (measured by the lag time in CD assay) and progression and severity of atherosclerosis. It cannot be denied, however, that the oxidation indices and conclusions, which can be derived from CD assays, strongly depend on assay conditions (Figs. 1–4). For example, vitamin E can be ineffective

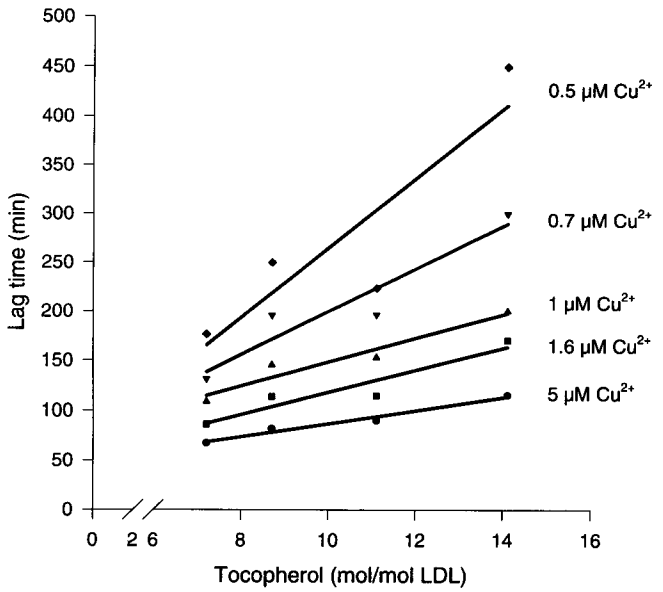


FIG. 6. Effect of α-tocopherol in the length of lag time measured with different Cu^{2+} concentrations. Low density lipoprotein (LDL) samples with different α-tocopherol contents (7.2, 8.7, 11.1, and 14.1 mol/mol LDL) were prepared and oxidized at 30°C with Cu^{2+} concentrations ranging from 0.5 to 5 μM. The graph shows that increase of lag time depends on both α-tocopherol content and Cu^{2+} concentration.

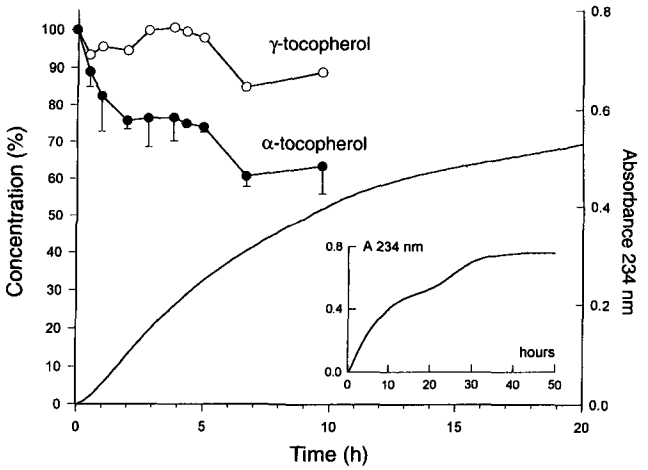


FIG. 7. Temporal relationship between loss of vitamin E and oxidation (conjugated diene increase) of low density lipoprotein (LDL) with 0.2 μM Cu^{2+}. Initial concentrations were 0.325 μM α-tocopherol, 0.016 μM γ-tocopherol, and 0.1 μM LDL.

(Fig. 7) or significantly increase lag time (Fig. 6), depending on the concentration of Cu^{2+} used as prooxidant. Much further work is needed to clarify whether oxidation indices measured *in vitro* are really suitable and important for predicting a risk.

ACKNOWLEDGMENTS

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Determinants of Antioxidant Status in Humans

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ABSTRACT: Antioxidant status in humans reflects the dynamic balance between the antioxidant system and prooxidants and has been suggested as a useful tool in estimating the risk of oxidative damage. This paper reviews determinants of antioxidant status such as diet including antioxidant nutrient and nonnutrient intake, absorption and bioavailabilty, dietary components such as polyunsaturated fatty acids and transition metals, food storage and processing, chemical form, chirality and formulation of supplemental compounds and alcohol intake; environmental factors such as pollutants, ultraviolet radiation and smoking; injury and disease, medications and other medical treatments such as radiation; strenuous exercise; and physiological stage or conditions such as those in premature babies and the elderly. It is proposed that, in addition to current focus on tissues, the antioxidant status of digesta should be considered because of its effect on specific tissues and potential health implications.

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Oxidation and production of free radicals are an integral part of human metabolism. Oxygen is the ultimate electron receptor in a closely linked electron flow system that produces energy in the form of ATP. Under certain conditions, the electron flow may become uncoupled, leading to production of free radicals, which are molecules with unpaired electrons and thus very reactive. Examples of free radicals include superoxide ($O^{\bullet}_2$), hydroxyl ($OH^{\bullet}$), peroxyl ($RO^{\bullet}_2$), alcoxyl ($RO^{\bullet}$), oxides of nitrogen ($NO^{\bullet}$, $NO^{\bullet}_2$), and thiyl ($RS^{\bullet}$). In addition, free radical reactions may be induced by reactive oxygen–containing species (ROS) generated in biological systems such as phagocyte activation, lipid oxidation, the arachidonate pathway, autoxidation of catecholamines, reduced flavins and hemoprotein, and iron-mediated reactions. Examples of ROS include singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl) (1–3).

Free radicals and ROS are either produced deliberately to serve important biological functions or accidentally. For example, activated phagocytes produce ROS and use them in the killing of some strains of bacteria and fungi. $O^{\bullet}_2$- plays a useful role in intercellular signaling and cell growth regulation. At high concentrations, however, free radicals and ROS react with nonradicals and can initiate adverse chain reactions such as lipid peroxidation. They also damage other important molecules, including proteins, carbohydrates, and DNA. Because of their high potential to damage vital biological systems, free radicals have been implicated as contributing factors to aging (4) and development of chronic disease, including heart disease and cancer (1–3,5).

Living organisms have developed complex antioxidant systems to control production and reduce damage from free radicals. This paper reviews briefly the antioxidant system in humans and discusses major factors determining its status.

Antioxidant system. The components of the antioxidant system may be classified on the basis of their origin as endogenous or dietary. Endogenous components include glutathione (GSH, also present in foods) and Se-glutathione peroxidase, Fe-catalase, NADPH, ubiquinol-10 (reduced coenzyme Q_{10}), Mn, Cu, Zn-superoxide dismutase, and uric acid. Metal-binding proteins include albumin (and albumin-bound thiols and billirubin), Fe- and Cu-binding proteins (transferrin, ceruloplasmin), and Fe-complex binding proteins (heptoglobin, hemopexin).

Antioxidants of dietary origin include tocopherols (vitamin E), ascorbate (vitamin C), vitamin A, carotenoids (ß-carotene, lycopene, lutein, and so forth), and Se (and other metals essential for the function of antioxidant enzymes). Many foods contain synthetic antioxidants (butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate, and tertiary butylhydroquinone) as additives to inhibit lipid peroxidation (6), which, however, may have antioxidant effects in the body.

Antioxidants may also be classified on the basis of other criteria such as solubility in lipid or water, proteins (enzymatic and nonenzymatic), or small molecules, among others. The function of individual antioxidants should be viewed in the context of a system with significant interdependence and additive or synergistic effects. For example, ascorbic acid and GSH may play a role in regeneration of vitamin E.

Antioxidant status. The balance between antioxidants and prooxidants in living organisms is defined as antioxidant status. This balance is dynamic and, in the human body, is tipped slightly in favor of oxidation (1), as evidenced by ongoing damage of DNA, lipids, carbohydrates, proteins, uric acid, and so forth. The body has adapted to this slight imbalance by developing repair mechanisms including ligases, nucle-

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Abbreviations: HDL, high density lipoproteins; H_2O_2 hydrogen peroxide; HOCl, hypochlorous acid; LDL, low density lipoproteins; $NO^{\bullet}$, $NO^{\bullet}_2$ oxides of nitrogen; O_2–, superoxide; $^1O_2$, singlet oxygen; OH, hydroxyl; PUFA, polyunsaturated fatty acids; RDA, recommended dietary allowance; $O^{\bullet}$, alcoxyl; $RO^{\bullet}_2$, peroxyl; ROS, reactive oxygen–containing species; $RS^{\bullet}$, thiyl; TBARS, thiobarbituric acid-reactive substances.

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ases, polymerases, proteinases, and phospholipases, to name a few. In addition, gradual and modest increase in oxidation induces production of endogenous antioxidants, thus providing a type of feedback-regulation mechanism (7).

Oxidative stress, a serious imbalance favoring oxidation, is said to result from excessive production of ROS and free radicals or from weakening of the antioxidant defense system (7). Severe oxidative stress can cause cell damage. Thus prevention of oxidative stress may be important for good health and prevention of disease.

Factors affecting antioxidant status. Antioxidant status is determined by factors affecting the antioxidant system and/or production of free radicals and ROS. Major factors affecting antioxidant status will be grouped as follows: diet, food, and alcohol; environment; injury and disease; physiological stage, exercise, aging, and other factors.

Diet, food, and alcohol, more than any other factors, directly affect both the antioxidant system (by supplying dietary antioxidants and cofactors of endogenous antioxidants) and production of free radicals and ROS [especially polyunsaturated fatty acid (PUFA) and alcohol]. In contrast, the major direct effect of other factors is on production of free radicals and ROS, with possible indirect effect on production of endogenous antioxidants by feedback mechanisms (7) or by reducing appetite and absorption of dietary antioxidants (for example injury and disease).

Diet, food, and alcohol: dietary antioxidants. Major components of the antioxidant system derived from the diet include tocopherols and tocotrienols (vitamin E), ascorbate (vitamin C), vitamin A, and its precursor, β-carotene. Other nutrients are essential for normal function of endogenous systems; minerals Cu, Mn, Zn, Se, Fe, and the vitamin riboflavin are important cofactors of antioxidant enzyme systems. In addition, phytochemicals, not recognized as nutrients (carotenoids such as lycopene, lutein, astaxanthin and others, bioflavonoids, phytosterols, and so forth), may play important antioxidant roles in the body (8).

Tissue levels of dietary antioxidants are affected directly by the amount and food composition of the diet (9,10) and by supplementation. GSH and uric acid may also be affected by diet. In contrast, dietary control of antioxidant enzymes and proteins is limited, except for deficiency of metal cofactors or serious malnutrition. Optimal intake of dietary antioxidants such as vitamin E is not known. Recent epidemiological studies indicated that risk of heart disease was lower in men and women taking 100 IU or more of vitamin E for more than one year (11,12). This level is several-fold higher than the recommended dietary allowance (RDA). Some current major clinical trials evaluate 300 and 400 IU/day (Hennekens C.H., and Yusuf, S., personal communications). For phytochemicals, there are no established RDA, and optimal intake is not known.

Chemical form, chirality, absorption, bioavailability, biochemical interactions between dietary antioxidants, and other factors affect antioxidant status. For example, dietary fat affects absorption of vitamin E and β-carotene (13,14). Similarly, high dietary levels of β-carotene affect absorption of lutein and possibly other carotenoids. Malabsorption may be caused by disease and drugs and will be discussed later. The d-α-tocopheryl forms (RRR) of vitamin E have higher bioavailability than the corresponding dl (all-racemic) forms (15,16). RRR-α-tocopherol is preferentially secreted in blood and incorporated into tissue in comparison to all-rac-α-tocopherol and non-α-tocopherols, namely β, γ, and δ (16).

Other dietary components. Nonantioxidant dietary components, especially those that can be oxidized or can promote oxidation, may have a major effect on antioxidant status. For example, high levels of PUFA are known to increase the requirement of vitamin E (17), which may be used to reduce formation of peroxides and other oxidation products. Other dietary components such as Cu and Fe are major promoters of free radical reactions. Reaction of Fe salts with H_2O_2, known as the Fenton reaction, yields the extremely reactive HO• (1). Phenolic phytochemicals such as isoflavonoids, which have been shown to have lipid antioxidant properties and may have beneficial health effects, can also promote damage of DNA and proteins by reducing metal ions such as Fe and oxidizing in their presence to generate H_2O_2 and $O^{\bullet}{}_2^-$ (18).

Food processing. Storage, handling, processing, and cooking of food can result in oxidative damage (5). The degree of damage and resulting oxidation products depend on a variety of factors, including temperature, exposure to light and air, amount and saturation of lipid material, presence of oxidation promoting metals, and other factors. Peroxides and prooxidants in processed foods affect antioxidant status.

Alcohol. Modest consumption of alcohol appears to cause desirable increase in high density lipoprotein (HDL) (19) and reduction in platelet aggregation (20); some alcoholic drinks, especially wine, supply phytochemicals (isoflavonoids, tannins, and so forth) having antioxidant effects including inhibition of low density lipoproteins (LDL) oxidation (18,20). Alcohol consumption, however, has been associated with increased liver damage and increased risk for chronic disease, especially some forms of cancer. It is now recognized that alcohol causes formation of acetaldehyde, which is hepatotoxic, not only by the main alcohol dehydrogenase mediated reaction, but also by an accessory microsomal pathway that generates oxygen radicals. Depressed glutathione levels, inactivation of some enzymes, detection of exhaled ethane and pentane, reduction in iron in the liver, and increased thiobarbituric acid-reactive substances (TBARS) may be the result of free radical production (2,21). It was reported recently (22) that plasma ascorbic acid, α-tocopherol, and Se were lower in men consuming even modest amounts of alcohol compared to controls, while malondialdehyde was higher, indicating an adverse effect on antioxidant status. Alcohol may also affect antioxidant status by reducing food intake and by altering metabolism of key nutrients, thereby promoting deficiencies as well as toxic interactions with food components such as β-carotene (21).

Environment. Air pollutants, tobacco smoke, and exposure to chemicals and ionizing radiation are major environmental factors initiating free radical reactions.

Air pollutants. The most damaging pollutants are nitrogen dioxide (NO_2), produced primarily from combustion in automobile engines, and ozone, produced mostly from NO_2 by photochemical reactions and from oxygen and gasoline vapors (22). NO_2 adds to double bonds and abstracts labile hydrogen atoms, initiating lipid peroxidation and production of free radical species. It also reacts directly with vitamin C and oxidizes it. Lipids from lungs of rats exposed to NO_2 had lower PUFA and indications of increased diene hydroperoxides, TBARS, and cholesterol epoxides, indicating autoxidation. Alveolar macrophages in rabbits exposed to NO_2 showed decreased resistance to viruses and reduced phagocytic activity; in rats it caused a change in arachidonic metabolism (2,23).

Ozone, although not a free radical, is a strong oxidant and may lead to free radical production by reacting with PUFA to form ozonides and by inducing autoxidation of lipids (24). It has also been suggested that the intermediate product of an ozone-olefin reaction, called the Criegee zwiterion, breaks down to hydroperoxide, which can initiate autoxidation, and aldehydes, which have been suggested to be inflammatory signals. Ozone reacts directly with vitamin E. Vitamin C also may be affected because it is believed to reduce the tocopherol radical. It has been shown that animals with low vitamin E status were easily injured by chronic ozone exposure and that vitamins E and C were protective. It was also reported that vitamin E was higher in the lungs of animals exposed to ozone, probably as defensive response (25).

Other environmental pollutants include halogenated hydrocarbons and pesticides. Although many chemicals do not spontaneously generate free radicals, their metabolism by the cytochrome P-450 enzyme system may cause free radical reactions (2).

Tobacco smoke. More than 1,000 chemical compounds are found in gas and tar fractions of tobacco smoke. NO_2, one of the major compounds in the gas phase, has been suggested to react with smoke olefins to form carbon-centered radicals. Alcoxyl radicals were also detected by spin trapping methods (26). In addition, tobacco smoke may increase free radical production indirectly by activating neutrophils. It has been suggested that nitric oxide, the precursor of NO_2, reacts with $O^{\bullet}{}_2^-$ to form peroxynitrite, which inactivates α-1-proteinase. The tar phase contains a semiquinone radical promoting formation of H_2O_2, which, along with transition metals, may inhibit the α-1-proteinase but at a slower rate. This inhibition may be related to increased production of elastase and development of emphysema (2,27). Several studies have shown that smokers have lower plasma levels of vitamins E, C, and β-carotene due to both lower intake and increased metabolic use. Even larger differences in favor of nonsmokers were reported for alveolar fluid (23,27,28). These findings and supporting epidemiological data provided impetus for evaluating the effect of supplemental antioxidants on the incidence of lung cancer. The Finnish study (a major intervention study), however, failed to show any reduction of lung cancer in heavy, long-term male smokers receiving supplemental β-carotene (25 mg/d) and/or dl-α-tocopheryl acetate (50 IU/d); actually, mortality from lung cancer was 18% higher in the β-carotene-supplemented group (29). No conclusive explanation for these unexpected results is available. It has been proposed that cancer development may have been progressing for several years, and antioxidants tested could not reverse this process. In addition, the adverse β-carotene effect may have resulted from its interaction with the high alcohol intake of a large number of participants (21).

Ionizing radiation. Sunlight and especially its ultraviolet component, initiate free radical reactions within cells, as evidenced by induction of lipid peroxidation (30) and suppression of replicative DNA synthesis due to DNA damage (31). Artificial sources of light with similar spectrum and other sources of ionizing radiation produce similar effects (32). It has been suggested that skin cancer and skin aging are associated with damage from free radical reactions. Antioxidants, and particularly dietary or topically applied vitamin E, inhibited lipid peroxidation and immunosuppresion and restored thymidine incorporation in mice suggesting that ultraviolet radiation affected the antioxidant status (33,34).

Injury and disease. Injury and disease affect the antioxidant status by reducing the supply of dietary antioxidants and by increasing production of ROS and free radicals. Tissue damage is caused by a variety of factors, including infection, trauma and burns, ultrasound, X-ray and other radiation, surgery and ischemia, reperfusion, exposure to high oxygen levels, excessive exercise, medications, toxins, and so forth. Chronic disease such as heart disease, cancer, arthritis, cataracts, diabetes, and others may also cause tissue damage and inflammation. In acute injury and infection, production of ROS, free radicals, and mediators of inflammation, is a secondary phenomenon, yet possibly very important in causing additional damage. In contrast, oxidative damage has been suggested as major causative factor for chronic disease with clinical symptoms and secondary increase in ROS and free radicals appearing much later (1,4,7).

Tissue injury causes free radical formation by releasing heme proteins and transition metals (mainly Fe), activation or increase of enzymes involved in free radical–generating reactions, increased utilization of antioxidants, and disruption of electron transport chains (1,2,35). In addition, tissue injury is associated with increased production of mediators such as prostaglandins, leucotrienes, interleukins, interferons, and tumor necrosis factors leading to inflammation. Activated macrophages are important sources of free radicals (35).

Diseases affecting the supply of dietary antioxidants. Many disease conditions and drugs depress appetite and thus affect the supply of dietary antioxidants and cofactors of endogenous antioxidants. Other diseases, such as cholestasis, fat malabsorption syndrome, short bowel syndrome, abetalipoproteinemia, and cystic fibrosis impair absorption or transport of vitamin E and other dietary antioxidants, leading to

serious deficiencies (36,37). Treatment with a water-soluble form of vitamin E improved vitamin E status in cholestatic children (37) and in a patient suffering from short bowel movement (38). Some diseases are associated with intestinal inflammation, fungi and bacterial infections, and diarrhea and thus affect absorption of nutrients. For example, lower blood levels of some vitamins and minerals in patients with acquired immunodeficiency syndrome may be be due in part to impaired absorption (39). Deficiency of key antioxidant components, such as vitamin E and Se, increases susceptibility to infection, which may be related to the antioxidant status (40,41).

Injury and disease associated with iron-catalyzed radical reactions. Some disease conditions such as sickle cell anemia, other hemoglobinopathies, iron overload disease, and cerebrospinal hemorrhage, and others causing tissue damage and hemolysis, release Fe and other transition metals, which catalyze free radical reactions, producing peroxy radicals (1,2,42,43). Similarly, premature babies and even normal-term babies have iron forms capable of catalyzing free radical reactions (44). Exposure of premature babies to high concentrations of oxygen causes retinopathy, probably due to oxidative damage.

Ischemia and reperfusion. Ischemia forces cells into anaerobic metabolism and causes uncoupling of the electron transport system, release of catecholamines, and production of xanthine oxidase. Reoxygenation results in catalysis of purines to uric acid and production of $O^{\bullet}_2$- and H_2O_2, production of other radicals from uncoupled electron transport, autoxidation of catecholamines, and activation of phagocytes. In addition, release of hemoglobin from damaged cells accelerates free radical production (45).

Chronic disease. For chronic diseases such as heart disease, cancer, and cataracts, major attention has been focused on the strong contributory role of oxidative damage from ROS and free radicals. For heart disease, a current hypothesis suggests that oxidation of LDL, probably mediated by monocytes and smooth muscle cells, and its uptake by the macrophage scavenger receptor to form foam cells, is a major contributor to atherosclerosis (46). Carcinogens and tumor promoters give rise directly to free radical reactions (47). Similarly, these reactions have been implicated in the development of arthritis, cataracts, emphysema, and other chronic diseases (1,2,4,7). The effect of chronic disease, however, on antioxidant status, is secondary and results primarily from tissue damage and activation of phagocytes (1,4).

Other diseases, medical treatments, and medications. In addition to reducing appetite, causing diarrhea, or otherwise affecting absorption and utilization of nutrients, other diseases, including infectious diseases, affect the antioxidant status by causing cell damage or by causing inflammation and increased production of phagocytes, as described above. Drugs may have direct effects on absorption, transport, and utilization of nutrients (48,49).

Radiation treatment causes lipid peroxidation and other free radical reactions similar to those produced by environmental radiation. Drugs and toxins may cause free radical reactions directly or through metabolites produced by the cytochrome P-450 detoxification system.

Physiological stage, aging, exercise, and other factors. Premature babies are susceptible to oxidative stress due to immature intestinal (poor absorption of nutrients) and lung function, hemolysis, and iron-mediated free radical production, as discussed above. At the other end of the spectrum, oxidative damage has been suggested as a contributory factor in the aging process (3). Aging has a secondary effect on antioxidant status because it is associated with higher incidence of inadequate nutrient intake, decline of organ function, weaker immunity, chronic disease, and higher intake of medications (3,48,49).

Strenuous exercise was reported to produce biochemical reactions similar to acute infection (50) and cell damage and thus it induces similar free radical reactions as evidenced by increased indices of oxidation including breath pentane (51,52).

It is believed that other factors which cause major emotional and/or physical stress, such as fear, grief, and physiological and hormonal changes, can affect the antioxidant status either by reducing appetite and/or by causing biochemical reactions in the body, which cause production of free radicals.

Evaluation of antioxidant status. Antioxidant status can be evaluated by direct measurements of components of the system, such as glutathione, tocopherol, ascorbate, and others, or, more commonly, indirectly by measuring oxidative stress (53,54). Because free radicals and ROS are extremely reactive and have a very short life, they can be measured directly only by electron spin resonance. In practice, the electron spin trap is used to measure radical spin adducts. Most other methods measure adducts, endproducts, or other compounds that are indicators of oxidative damage. These include lipid hydroperoxides, oxidized DNA, TBARS, malondialdehyde, conjugated dienes, oxidized LDL, volatile hydrocarbons, eicosanoids, H_2O_2, and others (54,55).

Due to strong preference for noninvasive methods for humans, the focus has been on indicator products present in breath (volatile hydrocarbons, H_2O_2), urine (TBARS, malondialdehyde, eicosanoids), and blood (TBARS, oxidized LDL, H_2O_2, glutathione, and others).

Antioxidant status of the digesta. Currently, antioxidant status is almost exclusively considered in the context of blood and other tissues. It may be appropriate, however, to consider the digesta as a major component. From the time of ingestion of food to excretion of fecal material, digesta undergoes major physical and chemical changes, including acidification, complex enzymatic and other reactions, emulsification of lipids with bile salts, and so forth. In addition, microflora in the gut is an integral part of digestion and may influence absorption. Microflora is affected by diet composition, disease, medication, and other factors. The many complex reactions occurring in the gut could lead to production of free radicals and ROS, which may have a direct effect in the development of digestive cancers, intestinal inflammation, and possibly

other diseases. In addition, harmful endproducts are absorbed and can cause tissue damage. Current concepts of absorption and bioavailability may not fully reflect the utility of various compounds and formulations. For example, rats fed equimolar amounts of α- or γ-tocopherol showed a significantly higher concentration of α in the blood, while most of the γ was found in the feces. γ-Tocopherol reduced plasma lipid peroxidation more than did α (56,57). Its potential for reducing peroxides and mutagens in the feces is being evaluated. In addition, γ-tocopherol was suggested as superior for detoxification of NO_2 (58). Yet γ-tocopherol is considered of little value as a form of vitamin E (51). Similarly, other compounds that are not absorbed or are absorbed and appear in urine without known biological function may have biological functions, including antioxidant function (59); this function may be, in part, in the digesta or the urinary tract.

Changing antioxidant status with nutritional supplements. Although there is substantial agreement that a significant imbalance in the antioxidant status favoring prooxidants may be harmful, benefits of altering the balance in favor of antioxidants remain controversial. Some researchers suggest that boosting the antioxidant status has no benefits and can be harmful (60,61). A prooxidant role of vitamin C has been suggested, especially in conditions of impaired iron metabolism, such as hemochromatosis or transfusion therapy (62). A prooxidant effect of vitamin E on LDL oxidation has been suggested from *in vitro* data (63) but prooxidant effect *in vivo* has not been shown. It has also been suggested as a promoter of initiated skin tumors in mice when applied topically (64). Proposed beneficial effects of supplementary antioxidants have been based on a large number of epidemiological studies that showed that high intake of fruits and vegetables (65,66) was associated with significantly lower risk for disease, especially heart disease and cancer. Furthermore, supplemental vitamin E, in excess of the RDA, has been suggested to reduce risk of heart disease (10,11). Fortunately, a large number of intervention clinical studies are now in progress and will help our understanding of the antioxidant status in health and disease.

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Antioxidants, Lipoprotein Oxidation, and Arterial Function

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ABSTRACT: Lipoproteins have an important influence on arterial tissues. High levels of low density lipoproteins (LDL) may inhibit arterial function in terms of the release of nitric oxide from the endothelium. This inhibition is enhanced by the oxidation of these lipoproteins which may occur during the development of atherosclerosis. Many of these effects are due to lipid oxidation products. The impairment of the release of nitric oxide and vasodilatation may be partially attenuated by antioxidants. LDL may also enhance the activity of blood platelets, especially when they are mildly oxidized. Oral administration of antioxidants may decrease the activation of platelets to a limited extent. Lipid peroxides may play a role in these processes. *Lipids 31*, S-83–S-85 (1996).

Oxidation of lipoproteins is considered to occur primarily in the arterial wall, although low levels of lipid peroxides are found in plasma associated with both low and high density lipoprotein (LDL and HDL, respectively) fractions (1). Plasma lipid peroxides may arise from release of slightly oxidized LDL from the subendothelial region of fatty streaks or more advanced plaques. Alternatively, they may arise from intestinal absorption of lipid peroxides in the diet. The endothelium may be exposed on the luminal side to low concentrations of these oxidation products, whereas on the abluminal surface, greater exposure to these cytotoxic molecules may occur in areas affected by atherosclerosis.

One of the principal properties of the endothelium is its ability to regulate vascular tone, mainly by release of nitric oxide (NO) in response to the pulsatile action of circulation or through receptor-mediated processes by specific agonists, some of which may be released by activated platelets. NO is biosynthesized from L-arginine by the action of NO synthase, which has several forms, mainly represented by its constitutive form in the endothelium, and which is regulated by intracellular calcium concentrations. Release of NO has two important effects: (i) relaxing arterial smooth muscle, thereby regulating arterial tone and influencing blood pressure, and (ii) inhibiting of platelet activation at the endothelial surface. Both these effects are achieved by an increase in intracellular cyclic GMP concentrations as a result of a direct interaction of NO with guanylyl cyclase.

There is now ample evidence that in atherosclerotic vessels there is a reduced ability of the artery to respond to agonists that evoke relaxations, e.g., acetylcholine and substance P. This may be observed by angiographic measurements of coronary vessel diameter in response to these agonists. This impairment of relaxation often precedes detection of occlusion of vessels using angiography. We found that at least part of this impairment may be caused by LDL (2). In organ bath studies, we showed that native LDL (nLDL) will partially and reversibly inhibit relaxation of rabbit aortic rings to acetylcholine or calcium ionophores. Subsequently, it has been shown that impairment of relaxation occurs in hypercholesterolaemia and that impairment can be reduced by cholesterol-lowering drugs, using forearm blood-flow measurements by plethysmography (this shows effects on resistance vessels) and ultrasound (large vessels) to demonstrate impairment (3,4). If LDL is oxidized with Cu^{2+}, impairment is intensified, especially after preexposure of the aortic rings to oxidized LDL (oxLDL) for short periods (5). Some samples of oxLDL produce an irreversible impairment of relaxation. Similarly, inhibition by oxLDL is observable in smaller coronary vessels, which may be classed as resistance vessels (6). In one study in atherosclerotic vessels, impairment of relaxation was found to be paradoxically associated with an increase in release of NO, possibly indicating a compensatory or protective adaptation (7). We have shown, however, that release of NO from cultured endothelial cells is decreased by oxLDL and, to a lesser extent, by nLDL when bioassayed with target fibroblasts (cyclic GMP) sensitive to NO. There is also an increase in the activity of superoxide dismutase, which should protect NO from reaction with superoxide anion and the consequent formation of peroxynitrite. Many of these actions of oxLDL can be demonstrated to varying extents, to be due to the products of lipid oxidation: lysophosphatidylcholine, hydroperoxy and hydroxy fatty acids, as well as cholesterol oxides, e.g., 7-keto cholesterol (8–11). Lysophosphatidylcholine appears to be the most potent.

These actions of lipoproteins may be decreased by antioxidants. HDL are known to have antioxidant properties and can

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Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; mmLDL, minimally oxidized LDL; nLDL, native LDL; NO, nitric oxide; oxLDL, oxidized LDL.

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reverse effects of oxLDL, but have little effect on nLDL (12). By giving probucol orally to human donors and then oxidizing isolated LDL in the presence of Cu^{2+}, impairment of relaxation is reduced. Probucol and other antioxidants (except HDL), will not reverse impairment of relaxation by oxLDL in the organ bath, since oxidation has already occurred. Both probucol and ascorbate, however, will reverse inhibitory effects of nLDL using the same procedure. Animal experiments have also shown some beneficial effects of antioxidants, and it was found that dietary supplementation with α-tocopherol in cholesterol-fed rabbits reduced impairment caused by the cholesterol diet. We have shown recently that probucol not only reverses atherosclerosis seen in adult Watanabe hereditary hypercholesterolaemic rabbits, but also partly restores the severe loss of vasodilatory properties seen in the vessels of these animals (13). We are currently studying effects of antioxidants in human subjects.

Finally, lipoproteins and antioxidants are reported to have effects on blood platelets (14). In our hands, nLDL has a mildly inhibitory effect on platelets, which may be associated with binding of nLDL to fibrinogen binding site on the glycoprotein IIb/IIIa complex on the platelet plasma membrane. These effects may be demonstrated by using conventional platelet aggregometry and also by flow cytometry using fluorescent antibodies (15). LDL that have been severely oxidized in the presence of Cu^{2+} also inhibit platelets because some oxidation products such as lysophosphatidylcholine are known to inhibit platelet activation. Minimally oxidized LDL (mmLDL), however, containing lipid peroxides (40–50 nM), slightly above the range seen in normal plasma, increase sensitivity of platelets to conventional platelet agonists. Addition of 15-hydroperoxy-eicosatetraenoic acid to nLDL to achieve the same peroxide concentration as mmLDL also has the same effect. If the concentration of 15-hydroperoxy-eicosatetraenoic acid is increased tenfold over those in mmLDL, the peroxides become inhibitory.

The presence of nLDL, mmLDL, or oxLDL has only slight effects on the sensitivity of platelets to inhibition by NO, reducing their sensitivity to NO. In complete contrast, hydrogen peroxide increases the sensitivity platelets to NO by two orders of magnitude (16). Lipid peroxides do not interfere with these interactions.

The literature concerning effects of antioxidants of platelet activation is conflicting. In recent studies, we have found that there is a small, but in some cases significant, decrease in the sensitivity of platelets to agonists ADP, collagen, and arachidonic acid and an increased sensitivity to the platelet inhibitor prostaglandin E_1 following supplementation of human volunteers with tocopherol, ascorbate, and β-carotene. This work complemented earlier observations of Salonen *et al.* (17) that patients with low antioxidant status and high plasma lipid peroxide concentrations had platelets that were more sensitive to platelet agonists and more likely to release platelet secretory proteins such as β-thromboglobulin.

These studies indicate that lipid peroxides and antioxidants have opposing effects on both endothelium and platelets and may influence the course of events in atherosclerosis/thrombosis.

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The Effect of Modest Vitamin E Supplementation on Lipid Peroxidation Products and Other Cardiovascular Risk Factors in Diabetic Patients

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ABSTRACT: Among many factors, elevated lipids and lipid peroxide levels in blood are major risk factors in the development of cardiovascular disease in diabetic patients. This study has examined whether oral supplementation of vitamin E, an antioxidant, has any effect on blood lipid peroxidation products (LP) and lipid profile of diabetic patients. Thirty-five diabetics(D) were supplemented with DL-α-tocopherol (E) capsule (orally, 100 IU/d) or placebo (P) for three months in double-blind clinical trials. Plasma E was analyzed by HPLC and LP by the thiobarbituric acid-reactivity; serum lipids by auto-analyzer. Data were analyzed using paired *t*-test and Wilcoxon Signed Rank Test. Vitamin E supplementation significantly lowered LP and lipid levels in diabetic patients; there were no differences in these parameters after P supplementation. There were no differences in the duration of diabetes and ages of D between P- and E- supplemented groups. This study suggests that vitamin E supplementation significantly lowers blood LP and lipid levels in diabetic patients.
Lipids 31, S-87–S-90 (1996).

It has been proposed that some of the complications of diabetes are associated with the increased activity of reactive oxygen species and accumulation of lipid peroxidation products (1–3). The evidence for increased lipid peroxidative damage in diabetes has been reported as far back as 1979 by Sato *et al.* (4). These authors reported that the average level of lipid peroxide in plasma is higher in diabetic patients than in normal people, and that diabetic patients with angiopathy had higher lipid peroxide levels than other diabetic patients. Because atheroma contains high levels of lipid peroxide, Sato *et al.* (4) further suggested that high levels of lipid peroxide in plasma may cause an increase in lipid peroxidation levels in the intima of the blood vessel which may then initiate the formation of atherosclerosis. Subsequent studies from other laboratories have confirmed that blood of both Type-I and Type-II diabetics as well as diabetic rats has elevated levels of lipid peroxidation products (5–16). Increased levels of lipid peroxides and LP in the blood can attack the blood vessels and cause cellular injury (17). Even slight injury to the endothelial cells of the artery or oxidative modification of lipoproteins has been implicated in atherogenesis (1,17). The present study was undertaken to examine the effect of daily supplementation with a modest dose of antioxidant vitamin E (100 IU) on blood level of LP and lipids in Type-I (IDDM) diabetic patients.

MATERIALS AND METHODS

Diabetic patients who agreed to participate in this study were asked to come to the diabetic clinic after overnight fasting and before taking any insulin. They were told to bring their insulin and syringes for use after drawing the blood. All patients were provided with free breakfast at the medical center cafeteria after the blood drawing. The routine check-up of the patients was done after they had eaten their breakfast. Alternate diabetic patients visiting the clinic were assigned to oral vitamin E (100 IU) or placebo capsule daily until the next visit (after three months). There was no control on the diet of these patients. Physicians and researchers in the laboratory did not know which patients were on vitamin E or placebo in the study. Only the nurse in charge knew about the assignment of patients to vitamin E or placebo. Thirty-five diabetics were enrolled in this study. Fasting blood was collected before the start of and after the period of vitamin E or placebo supplementation from each diabetic. All diabetic patients included in this study had Type-I diabetes (insulin-dependent). African-Americans were grouped as blacks and Caucasian-Americans were grouped as whites. All biochemical analyses were done immediately after blood collection. Plasma vitamin E was measured by high-performance liquid chromatography (13). Malondialdehyde (MDA) was measured by the thiobarbituric acid reactivity (18). Serum triglycerides, total cholesterol, and high density lipoprotein- and low density lipoprotein-cholesterol were determined by auto-analyzer. Before the code opening, out of 35 diabetics enrolled, six were deleted for non-compliance for reasons such as lost bot-

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Abbreviations: LP, lipid peroxidation products; MDA, malondialdehye.

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TABLE 1
Effect of Oral Vitamin E or Placebo Supplementation (Suppl.) on Plasma Vitamin E Levels in Type-I Diabetic Patients[a]

	Baseline (D_1)	E-Suppl. (D_1 + E)	Baseline (D_2)	P-Suppl. (D_2 + P)
n	13	13	16	16
α-Tocopherol (nmole)				
per mL	16.3 ± 1.1[b]	29.1 ± 1.7[c]	18.5 ± 1.2	17.6 ± 1.8
per μmole phospholipid	10.7 ± 0.3[b]	19.5 ± 1.0[c]	11.4 ± 0.7	11.1 ± 0.7
γ-Tocopherol (nmole)				
per mL	5.5 ± 0.7[b]	2.0 ± 0.3[c]	6.8 ± 0.8	5.5 ± 1.1
per μmole phospholipid	2.5 ± 0.3[b]	1.4 ± 0.2[c]	4.1 ± 0.5	3.3 ± 0.5

[a]Values are mean ± SE; n is the number of patients in each diabetic group. Differences in values between different superscript letters are significant ($P < 0.0001$).

tle, lost track, started taking another medicine, or patient was found to have thyroid disorder. After the analyses and code opening, there were 13 D on E and 16 D on P supplementation for which data were analyzed using paired *t*-test and Wilcoxon Signed Rank Sum test.

RESULTS

The distribution of patients in terms of age, daily insulin use, duration of diabetes, sex, race and supplementation was similar between vitamin E and placebo supplemented diabetic groups. Table 1 shows that plasma α-tocopherol level expressed as per ml or per total phospholipid was markedly higher in diabetic patients supplemented with vitamin E compared with baseline values. However, there was no effect on vitamin E level in placebo supplemented diabetic patients compared with baseline values. The lower γ-tocopherol level in vitamin E-supplemented group suggests that higher α-tocopherol levels are due to its exogenous supplementation.

Table 2 shows that MDA level was significantly lower ($P < 0.05$) in diabetic patients after supplementation with vitamin E but not in patients supplemented with placebo, compared with respective baseline levels. The effect of vitamin E on plasma LP was observed even after its normalization with total phospholipid. There was no difference in the baseline plasma MDA levels between vitamin E or placebo supplemented groups.

Table 3 shows that vitamin E supplementation significantly lowered ($P < 0.03$) the plasma triglyceride levels in contrast to placebo-supplementation when compared with the respective baseline values. However, there was no effect of vitamin E or placebo supplementation on the total, high density lipoprotein- or low density lipoprotein-cholesterol of diabetic patients. There was also no difference in the mean insulin dosage intake by patients between before the start (D_1, 0.89 + 0.1; D_2, 1.0 + 0.1) and during the study (D_1 + E, 0.86 + 0.1; D_2 + P, 0.97 + 0.1) in both the groups.

DISCUSSION

The elevated level of lipid peroxides can cause oxidative injury to blood cells, crosslinking of membrane lipids and proteins (19,20), destabilization of membrane lipid organization (21–24), accelerated cellular aging (19,20,25), prostacyclin/prostaglandin imbalance and vasoconstriction (26). The level of LP is increased in plasma and other tissues of rats with alloxan- or streptozotocin-induced diabetes and in both Type I and Type II diabetic patients (4–16). The increase in plasma lipid peroxide levels of diabetic rats disappeared when diabetic rats were supplemented with 90 IU vitamin E/kg diet for 10 wk (27) or with 500 IU vitamin E/kg diet for 4 wk (28). However, there is no study that has examined the effect of vitamin E supplementation on plasma level of LP in diabetic patients. The present study has found a significant reduction in the level of LP after vitamin E supplementation in Type-I diabetic patients. This suggests that a modest dose of vitamin E supplementation can scavenge the lipid peroxidation processes in diabetics presumably caused both by the increased oxygen radical activity due to autoxidation of glucose and/or inactivation of antioxidative enzymes by the hyperglycemia (18,29).

The increase in plasma triglyceride levels was blocked in diabetic rats after vitamin E supplementation with intramuscular injections (30) or with dietary additions (27) and in dia-

TABLE 2
Effect of Oral Vitamin E or Placebo-Supplementation (Suppl.) on Plasma Lipid Peroxidation Products Level in Type-I Diabetic Patients[a]

Group	Baseline (D_1)	E-Suppl. (D_1 + E)	Baseline (D_2)	P-Suppl. (D_2 + P)
n	13	13	16	16
Thiobarbituric acid-reactivity (MDA)				
nmole/mL	0.44 ± 0.5[b]	0.34 ± 0.2[c]	0.38 ± 0.03	0.36 ± 0.02
nmole/μmole				
Total phospholipid	0.3 ± 0.04[b]	0.21 ± 0.02[c]	0.24 ± 0.02	0.24 ± 0.02

[a]Values are mean ± SE; n is the number of patients in each diabetic group. Differences in values between different superscript letters are statistically significant ($P < 0.05$); MDA, malondialdehyde.

TABLE 3
Effect of Oral Vitamin E or Placebo Supplementation (Suppl.) on Plasma Lipids Levels in Type-I Diabetic Patients[a]

	Baseline (D_1)	E-Suppl. (D_1 + E)	Baseline (D_2)	P-Suppl. (D_2 + P)
n	13	13	16	16
Total lipid (mg/mL)	4.15 ± 0.18[b]	3.69 ± 0.19[c]	4.36 ± 0.15	4.19 ± 0.33
Cholesterol (μmole/mL)	3.93 ± 0.21	3.92 ± 0.16	4.19 ± 0.22	4.23 ± 0.23
HDL-Chol (μmole/mL)	1.36 ± 0.07	1.44 ± 0.09	1.39 ± 0.07	1.44 ± 0.09
LDL-Chol (μmole/mL)	2.14 ± 0.16	2.15 ± 0.13	2.40 ± 0.18	2.34 ± 0.17
Triglycerides (μmole/mL)	2.91 ± 0.27[d]	2.26 ± 0.22[e]	2.76 ± 0.48	3.10 ± 0.64

[a]Values are mean ± SE; n is the number of patients in each diabetic group. Differences in values between different superscript letters, [b,c] ($P < 0.01$) and [d,e] ($P < 0.03$) are significant.

betic patients (31) supplemented with pharmacological doses of vitamin E (900 mg/d for 3 mon). The present study using a modest dose of vitamin E supplementation also found an inhibitory effect on triglyceride accumulation but not on total-cholesterol and low density lipoprotein-cholesterol in Type I (IDDM) diabetic patients. The decrease in the triglyceride may be related to an improvement in the fasting plasma glucose level, and could also be due to an increase in the activity of lipoprotein lipase similar to that has been observed in the vitamin E supplemented diabetic rats by other investigators (27,30).

This study suggests that vitamin E supplementation significantly lowers blood lipid peroxidation products and triglyceride levels and could be beneficial in reducing risk of cardiovascular disease in diabetic patients.

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Modulation of Antioxidant Enzymes and Programmed Cell Death by n-3 Fatty Acids

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ABSTRACT: Studies from our laboratory indicate that n-3 (fish oil, FO) lipids at 10% (w/w) in a nutritionally adequate, semipurified diet, and supplemented with equal levels of antioxidants, extended the life span of lupus-prone (NZB/NZW)F_1 (B/W) female mice as compared to n-6 (corn oil, CO) lipids. The early rise of autoimmune disease in CO-fed mice was closely linked to the loss of T-cell function. Both IL-2 production and IL-2 receptor expression were reduced due to the loss of naive T-cells and a rise in memory T-cells. Proliferative response to both mitogens and superantigens (staphylococcal enterotoxins A and B) was higher in FO-fed 6.5-mon-old mice. These changes paralleled decreased PGE_2 production by splenic cells from FO-fed mice.

Analysis of mRNA expression in different organs revealed differential effects of dietary lipids. In FO-fed mice, transforming growth factor β1 (TGF β1) expression was decreased in kidneys, but splenic tissues had higher expression of TGF β mRNA. As TGF β promotes programmed cell death (PCD), we studied the effects of CO and FO on PCD rates in lymphocytes. Both propidium iodide staining and DNA fragmentation were elevated in lymphocytes of FO-fed mice when compared to CO-fed mice of similar age. Also, increased PCD correlated closely with increased Fas gene expression. Thus, in addition to various other antiinflammatory effects, dietary FO appears to increase PCD and prevent accumulation of self-reactive immune cells in lymphoid organs. Further studies are required to dissect the pro- and antiinflammatory mechanisms associated with dietary n-3 and n-6 lipids in modulating autoimmune disorders or malignancy during aging.

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Fish oil (FO) supplementation has antiinflammatory effects in both humans and experimental animals and delays onset of autoimmunity, cardiovascular disease, and cancer, particularly when compared to diets relatively enriched in saturated fats of animal origin (1,2). Because of adverse effects of animal fats, consumption of vegetable oils containing both monounsaturated n-9 and polyunsaturated fatty acids (rich in 18:2n-6) is rising significantly in the United States (1–3). Though vegetable oils rich in n-6 fatty acids reduce the incidence and the severity of cardiovascular disease, these lipids (particularly hydrogenated fats) have been linked to proinflammatory effects and the rise of certain cancers, rheumatoid arthritis, and other autoimmune disorders during aging (4). Though n-6 lipids reduce serum cholesterol levels, they usually tend to elevate linoleic acid (18:2n-6) levels and arachidonic acid (20:4n-6) levels in the tissue phosphoglycerides. Corn oil (CO) is rich in linoleic acid, and its consumption increases production of free radicals and cyclooxygenase metabolites such as thromboxane A_2 and leukotriene B_4 (LTB_4). In contrast, FO contains highly polyunsaturated fatty acids, eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), which act synergistically to inhibit prostaglandin E_2 (PGE_2) production and are thus antiinflammatory (5). Because of these beneficial effects, marine oils, especially menhaden oil, are receiving increasing attention as a source of C_{20} and C_{22} carbon n-3 fatty acids.

Animals and diet. We and others have reported that reduced calories and/or low-fat diets preserve T-cell functions, decrease autoantibody production, and reduce immune complex-mediated glomerulonephritis in (NZB/NZW)F_1 (B/W) mice (6–8). B/W mice spontaneously develop a disease similar to human systemic lupus erythematosus (SLE), which has been used as an animal model to elucidate the pathogenesis of the disease (9,10). The majority of the immunological abnormalities associated with human SLE are also seen in B/W mice, including hypergammaglobulinemia (a result of generalized B-cell hyperactivity), production of autoantibodies against a wide variety of endogenous antigens, formation of immune complexes, and a variety of associated histopathological manifestations (e.g., glomerulonephritis) (9,10). Female B/W mice under normal feeding conditions die between 6–10 mon of age, while males die between 10–22 mon of age. Death usually occurs as a result of renal failure. During the past 25 years, we have utilized female B/W mice for dietary

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Abbreviations: B/W mice, (NZB/NZW)F_1 mice; CAT, catalase; CO, corn oil; CTLL-2, cytotoxic T-lymphocyte line 2; FACS, fluorescence activated cell sorting; FO, fish oil; GSH-Px, glutathione peroxidase; ICAM-1, intercellular adhesion molecule-1; IL-2, interleukin-2; LTB_4, luekotriene B_4; MDA, malondialdehyde; MRL/*lpr* mice, MRL mouse with a lymphoroliferative (*lpr*) gene; PCD, programmed cell death; PGE_2, prostaglandin E_2; PI, propidium iodide; SLE, systemic lupus erythematosus; SOD, superoxide dismutase; TBARS, thiobarbituric acid–reactive substances; TBHQ, *tert*-butylhydroquinone; TGF β, transforming growth factor β; TNF-α, tumor necrosis factor-α.

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studies because of early onset of disease and short life span, which we found responsive to dietary manipulations (11–15).

Though dietary supplementation with FO significantly extends life span and alleviates autoimmune symptoms in B/W and MRL mice with lymphoroliferative (*lpr*) gene (MRL/*lpr* mice) (11–18), molecular mechanisms associated with these beneficial effects have not been fully explored. Therefore we sought to determine effects of isocaloric CO- or FO-enriched diets fed *ad libitum* on immunological and molecular parameters associated with onset and progression of disease in B/W mice. We fed weanling B/W mice a nutritionally adequate, semipurified diet supplemented with either CO or FO (10% wt/wt) and equal levels of antioxidants (11). Both oils were supplemented with 2.5 g tocopherol (α and γ) and *tert*-butylhydroquinone (TBHQ)/kg of oil to prevent peroxidation, as recommended by the Fish Oil Test Material Program (National Institute of Alcohol Abuse and Alcoholism). Each diet therefore contained ~215 mg vitamin E/kg (vitamin E from oil and from vitamin mixture) (11).

Immunological studies. We observed that B/W mice become more susceptible to autoimmune disease when fed a CO-supplemented diet (vs. FO) and expressed significantly higher levels of serum anti-dsDNA antibody levels, a parameter known to serve as a highly prognostic marker for onset and severity of renal disease (17). When splenocytes were stimulated with optimal concentrations of mitogens and superantigens, a significantly higher proliferative response was noted in FO-fed mice (vs. CO). When spleen cells were cultured in the presence of Con A for 48 h, and interleukin-2 (IL-2) levels were determined by a bioassay utilizing cytotoxic T-lymphocyte line 2 (CTLL-2) cell line, IL-2 levels (μ/mL) were significantly lower ($P < 0.05$) in FO-fed mice (vs. CO) (17). Though the percentage of $CD4^+$ and $CD8^+$ cells in splenocytes from both CO- and FO-fed mice (FACS analysis) did not differ, a significant decrease in Ig^+ cells ($P < 0.05$) was noted in FO fed group (vs. CO) (17). Also, IL-2 receptor expression fluorescense activated cell sorting (FACS) was significantly increased in FO-fed mice (data not shown), indicating a delay in shift from naive to memory cells in a T-cell subpopulation. In fact, spleens from FO-fed mice had higher naive and lower memory T-cells (17).

We next sought to determine whether FO supplementation alters gene expression in various organs associated with onset and progression of autoimmune disease in B/W mice. We studied expression of both mRNA and protein levels for the oncogenes-c-*myc* and c-*ras*- in spleens, transforming growth factor β (TGF β) in both spleen and kidneys and antioxidant enzymes (catalase, CAT; glutathione peroxidase, GSH-Px; and superoxide dismutase, SOD) in livers (enzyme activity and mRNA) and kidneys (mRNA expression).

Densitometric analysis of total RNA from spleens by Northern analysis (to compensate for any loading differences, values are expressed in ratios ± SEM of c-*myc*, c-*ras* or TGF β to GAPDH) revealed lower expression of c-*myc* and c-*ras* mRNA in FO-fed (vs. CO) mice, while TGF β mRNA was significantly higher (Fig. 1). Furthermore, in FO-fed mice, protein products (Western blotting) of both oncogenes was significantly lower, and for TGF β it was significantly higher (17).

TGF β was initially characterized on the basis of its ability to induce a transformed phenotype in normal rat kidney fibroblasts in culture (19). TGF β are now recognized as multifunctional, regulatory factors influencing a remarkable array of physiological processes, including embryogenesis, hematopoiesis, angiogenesis, immune function, inflammation, myogenesis, osteogenesis, tissue repair, and remodeling (20). Hence, TGF β impacts virtually every organ system, and it may not be surprising that aberrant expression or regulation of these molecules has been implicated in numerous pathological conditions, including autoimmune disorders, glomerulonephritis, diabetic nephropathy, tumorigenesis, and immunosuppression associated with acquired immunodeficiency syndrome and malignancies (19–21).

TGF β not only affects a plethora of physiological processes, but also elicits diverse cellular responses and may have both inhibitory or stimulatory effects on the same cell, depending on cell type, state of differentiation, and context of other factors or signals in the cellular environment. In FO-fed mice, significantly elevated TGF β expression was observed at both mRNA and protein levels. Also, significantly lower oncogene-c-*myc* and c-*ras*- expression was noted, indicating

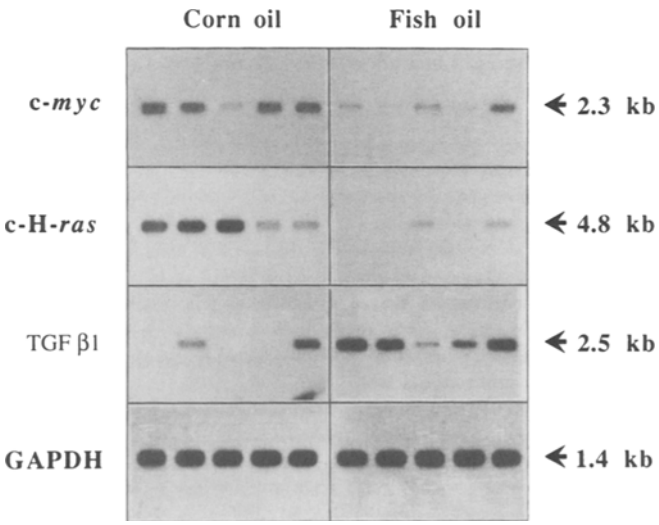


FIG. 1. Inhibition of c-*myc* and c-*ras*, and upregulation of transforming growth factor β (TGF β) mRNA expression by dietary fish oil supplementation (10% w/w) in spleens of 6.5-mon-old B/W mice (18). For Northern blot analysis, 20 μg of total RNA/lane was denatured, electrophoresed through 2.2 M formaldehyde/0.8% agarose gel, and electroblotted onto nitrocellulose. Blots were prehybridized, hybridized, washed, and autoradiographed as described (18). Autoradiographic exposure time was 36 h to 5 d. Blots were reprobed after stripping off previous labeling. GAPDH was used as an internal control and indicates equal levels of RNA loading in all lanes of the gel. Each lane represents an RNA sample from one animal.

that FO supplementation is immunosuppressive by increasing or by maintaining higher levels of the immunoregulatory molecule TGF β in splenic lymphoid cells (17). As Ig^+ cells and the serum anti-dsDNA antibody levels were significantly lower in FO-fed mice, this in turn may be due to higher levels of TGF β. Though it is a significant contribution to the field of nutrition that the source of dietary lipids can modulate TGF β expression, it has been shown previously in various *in vitro* studies that TGF β has immunosuppressive effects on immune cells, particularly on B-cell proliferation and immunoglobulin production (17).

TGF β and lupus nephritis. In contrast to its beneficial immunosuppressive properties, expression of TGF β has been reported to play a pathological role in various models of glomerulonephritis (22). Its role in either the initiation or progression of lupus nephritis is not known, however. Cellular proliferation is an important pathological element in the development of lupus nephritis. In an inflammatory process, local generation of TGF β may promote resolution of inflammation, but persistent production of TGF β, possibly in combination with cytokines, may promote accumulation of extracellular matrix (23). In CO-fed mice, a significantly higher histological score (vs. FO-fed mice, 1-fold lower, $P < 0.05$) and a higher frequency of perivascular infiltration of mononuclear cells was observed (12). Northern and Western blot analyses revealed significantly higher TGF β expression in kidneys from CO-fed mice as compared to FO-fed mice (12). Also, expression of fibronectin, one of the components of ECM, is significantly elevated in kidneys of CO-fed (vs. FO-) mice (12). Intercellular adhesion molecule-1 (ICAM-1), which is normally expressed on endothelial cells of glomeruli in kidneys and whose expression is increased by several fold in inflammatory conditions (24), was also significantly elevated in CO-fed mice (12). IL-1 and TNF α alter endothelial cell morphology and induce expression of adhesion molecules, including ICAM-1 (24). In kidneys from CO-fed mice (vs. FO) significantly lower IL-1, IL-6, and TNF α mRNA and protein levels were observed (14), indicating that higher levels of TGF β in kidneys might have modulated expression of these inflammatory cytokines. Taken together, these results indicate the dual nature of TGF β, behaving as a (beneficial) immunosuppressant in the spleen, but as a pathological agent in kidneys.

Autoimmunity and apoptosis. Within the immune network, TGF β mediates a negative feedback circuit inhibiting activation, proliferation, and effector functions. TGF ß decreases c-*myc* oncogene expression in lymphokine-stimulated T-cells and $CD4^+$ cells are affected more than are $CD8^+$ T-cells. Since TGF β modulates proliferation or function of every immune cell type, it is not surprising that its absence has devastating consequences as seen in TGF β knock-out mice (25,26). Accumulation of autoreactive B- and T-cells and severity of inflammation observed in some tissues of these mice raises the question of whether this pathology is somehow associated with aberrant cell death as a result of lower apoptosis or programmed cell death (PCD) in the immune system. Excess accumulation of cells can be due to either increased proliferation or decreased apoptotic cell death. In fact, TGF β expression has been associated with negative regulation of growth. It inhibits DNA synthesis in liver, mammary gland, and uterine endometrium (27). Enhanced expression of TGF β was found in castration-induced regression of prostate and in regressing tumors, suggesting its involvement in PCD (27). In primary cultures of uterine endometrial cells and of hepatocytes, TGF β induced cell death (28). In our dietary studies in B/W mice, spleens from FO-fed mice (vs. CO) expressed significantly higher levels of TGF β and lower levels of c-*myc*. Hence we studied the degree of apoptosis in the splenocytes from both CO- and FO-fed mice.

Reliable methods to assess apoptosis include endonuclease-mediated DNA fragmentation (ladder formation; extensive degradation of chromosomal DNA into oligomers of about 180 b.p.) and propidium iodide (PI) uptake and flow cytometric analysis of PI-stained apoptotic cells. Splenocytes from young FO-fed mice (vs. CO) did not show differences in PI staining, but old mice at 8–10 mon of age showed higher PI-positive cells (Fig. 2). Also in older mice, splenocytes exhibited higher DNA fragmentation (cultured for 18 h in the presence or absence of dexamethasone at 10^{-7} M; data not shown). Although differences in PCD appear to be small, *in vivo* physiological PCD may play a role in modulating autoimmune disease. Encouraged by these results, we have studied the effects of dietary CO and FO on expression of Fas, one of the positive modulators of apoptosis, in splenocytes.

The Fas antigen belongs to a receptor supergene family that includes TNFR, B-cell antigen CD40, NGFR, and T-cell Ag OX40 (29). Fas is broadly expressed on myeloid and lymphoid cells, on fibroblasts, liver cells, heart muscle, and in the ovarian follicle (29). The cytoplasmic domain of Fas probably interacts with signal transduction molecules such as G-proteins, kinases, and/or phospholipases since it has no apparent intrinsic enzymatic activity. Its importance is further emphasized by the fact that the Fas gene was found to be defective in mice carrying the lymphoproliferation (lpr) mutation, closely resembling human SLE (29). This defect in Fas expression leads to excessive numbers of self-reactive T-cells escaping from negative selection in the thymus, resulting in autoimmune disease. FO supplementation significantly increased or maintained higher levels of Fas gene expression in spleens from B/W mice (30). Also, Western blot analysis complemented gene expression, and significantly higher Fas protein levels were observed in the spleens of FO-fed (vs. CO-) mice (data not shown; 30). Thus, increased survival of B/W mice on FO may be due to higher apoptosis of autoreactive T-cells and elimination of self-reactive cells in the periphery. Further, higher PCD was also noted in lymphoid cells from both spleen and thymic tissues from MRL/*lpr* mice on calorie restriction (31).

Autoimmunity and antioxidant enzyme activity and gene expression. Since autoimmune diseases have multifactorial etiologies including genetic, hormonal, viral, and immunological factors, inability of the antioxidant defense system to

cope with oxidative stress has also been reported to contribute to disease progression (32,33). Unsaturated fatty acids can undergo free radical-initiated chain reactions. GSH-Px acts to protect cellular components such as unsaturated fatty acids from free radical oxidation by converting hydrogen peroxide, a potent endogenous free radical generator, to water. Though CAT plays a similar role as that of GSH-Px, GSH-Px is reported to be more important than CAT in hydroperoxide catabolism. Conversely, SOD detoxifies superoxide radicals, and all three enzymes play an important role in the host's antioxidant defense mechanism. Vitamin E, a lipophilic free radical scavenger, protects unsaturated fatty acids by terminating chain reactions involving fatty acid peroxyradicals. In-

TABLE 1
Effect of 10% (w/w) Corn Oil–Krill Oil–or Fish Oil–Based Diets on Activities of Hepatic Cytosolic Antioxidant Enzymes in B/W Mice (11)

Enzyme	Corn oil	Krill oil	Fish oil
Catalase[c]	59.1 ± 4.4[a]	529.9 ± 27.5[b]	472.0 ± 55.8[b]
GSH-Px[d]	25.5 ± 2.0[a]	98.7 ± 5.9[b]	106.0 ± 3.4[b]
SOD[e]	7.2 ± 0.3[a]	.9 ± 0.4[b]	10.2 ± 0.2[b]

[a,b]Values are mean ± SEM of 5 mice/group. Values with different superscript letters (a,b) in the same row are significantly different at $P < 0.05$ level as analyzed by Student's *t*-test with Bonferroni adjustment.
[c]Catalase activity is expressed as μmoles H_2O_2 reduced/mg protein/min.
[d]Glutathione peroxidase (GSH-Px) activity expressed as μmoles NADPH oxidized/g protein/min.
[e]Superoxide dismutase (SOD) activity is expressed in units/mg protein.

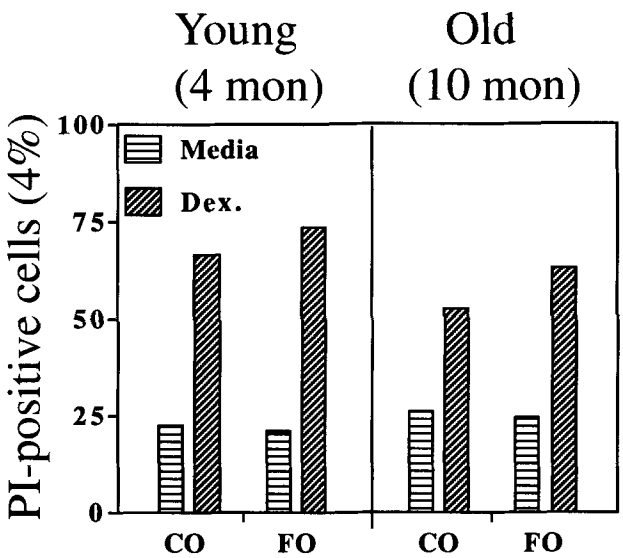


FIG. 2. Effects of dietary corn oil (CO) and fish oil (FO) (10% w/w) on programmed cell death (PCD) in splenocytes from young and old B/W mice as assessed by propidium iodide staining. PCD was lower in old mice on both diets as compared to that in young. Dexamethasone-induced PCD was higher in FO-fed mice (vs. CO) in both young and old mice. PI, propidium iodide.

adequate levels of vitamin E in the presence of low selenium uptake may also result in higher frequency of tumor development. We have demonstrated the importance of supplementing FO-containing diets with vitamin E. Both increased membrane vitamin E and decreased thiobarbituric acid-reactive substances (TBARS) generation was found by supplementing 500 IU of vitamin E with FO-containing diets (34). We have also studied the effects of dietary supplementation of CO and FO on free radical generation and the status of antioxidant defenses in livers and kidneys of B/W mice. TBARS in liver cytosolic fractions were significantly higher in CO-fed mice, indicating higher free radical generation (11). Hence we became interested in testing whether or not the degree of peroxidation in subcellular membrane lipids in autoimmune mice was due to defects in the antioxidant defense system. For this purpose, we analyzed enzyme activity for CAT, SOD, and GSH-Px in the liver cytosolic fraction, and mRNA expression for these enzymes in livers from CO- and FO-fed mice. Both enzyme activity (Table 1) and mRNA expression of these key antioxidant enzymes were significantly lower in the livers (Fig. 3) and kidneys (Fig. 4) of CO-fed mice (vs. FO) (11,12), indicating weaker antioxidant defense mechanisms in various organs in B/W mice fed a CO diet. As described earlier (11), the diets as well as the oils (CO and FO) were supplemented with equal levels of antioxidants, so that our results could not have been compromised because of differences in dietary antioxidant levels.

In summary, the use of highly refined FO with adequate antioxidant supplementation appears to delay onset of autoimmune disease when compared to n-6 (CO)-containing diets. FO also modulates PCD and enhances antioxidant enzyme mRNA levels and decreases free radical-induced tissue damage *in vivo*. Further studies at the molecular level are needed to dissect the protective mechanisms of FO.

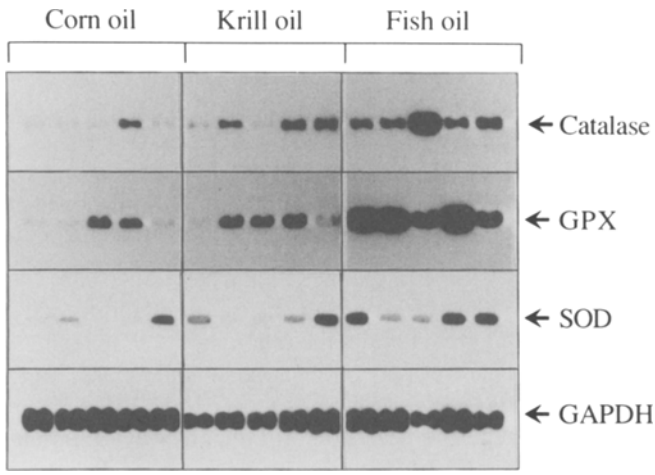


FIG. 3. Higher hepatic antioxidant enzyme gene expression (Northern blot) in 6.5-mon-old B/W mice fed a diet supplemented with CO or FO (10% w/w) (12). Autoradiographic exposure time was 8 d for the enzymes and 36 h for GAPDH. Similar to fish oil, krill oil is a source of n-3 lipids, and krill oil also increased (vs. CO) enzyme gene expression significantly. Each lane represents an RNA sample from one animal. Abbreviations as in Figure 2.

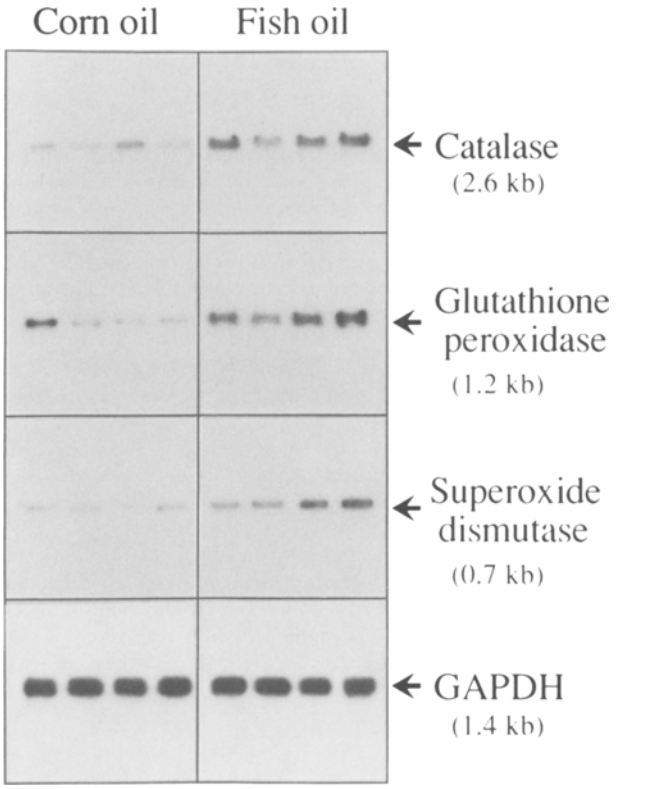


FIG. 4. Northern blot analysis of total RNA isolated from kidneys of 6.5-mon-old B/W mice fed a diet supplemented with CO or FO (10% w/w) (15). Autoradiographic exposure time was 4 d for the enzymes and 36 h for GAPDH. Each lane represents an RNA sample from one animal. Abbreviations as in Figure 2.

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In Vivo Labeling of Brain Phospholipids by Long-Chain Fatty Acids: Relation to Turnover and Function

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ABSTRACT: An experimental method and model are described to quantitate kinetics of *in vivo* incorporation of fatty acids (FA) into stable brain phospholipids. When a radiolabeled long-chain FA is injected intravenously in a rat, it rapidly equilibrates with brain FA–CoA, the precursor pool for phospholipids. As different labeled FA enter different *sn* positions of specific phospholipids, a combination of labels can be used to investigate roles of different phospholipids in brain function and structure. By taking into account dilution λ of specific activity of brain FA–CoA, compared with specific activity of FA in plasma, half-lives of FA in individual brain phospholipids can be calculated. Values for λ less than 0.02 suggest marked recycling, and give half-lives two orders of magnitude smaller than literature values. A half-life of arachidonate in phosphatidylinositol of 0.66 h (turnover = 105 %h) is consistent with active participation of this FA in phospholipase A_2mediated signal transduction.

Lipids 31, S-97–S-101 (1996).

Brain functional activity and structural integrity are related to half-lives of acyl groups within phospholipids (1,2). Until recently, these terms were estimated by injecting radiolabeled precursors intracerebrally and killing animals at different times thereafter to determine phospholipid radioactivity (3–5). Unfortunately, intracerebral injection damages brain tissue and provides half-lives that ignore recycling and/or *de novo* synthesis.

To overcome these limitations, we developed an *in vivo* method and model to quantify incorporation into and half-lives of fatty acids (FA) in brain phospholipids (6). An albumin-bound radiolabeled unacylated long-chain FA is injected intravenously (i.v.). Labeled and unlabeled FA in plasma are monitored until the animal is killed at 20 min and the brain is prepared for chemical analysis or quantitative autoradiography. Different FA tracers pulse label specific *sn* positions in different brain phospholipids.

MODEL AND RESULTS

In our model, we identify two experimentally-accessible brain compartments (Fig. 1): (i) an "unstable" final precursor FA (acyl)– CoA pool whose specific activity equilibrates rapidly with plasma specific activity, on the time-scale of an experiment; and (ii) a "stable" phospholipid/neutral lipid compartment in which FA turnover is slow compared with the time scale of an experiment. Fast kinetics of FA–CoA are consistent with evidence that its specific activity equilibrates with plasma unacylated FA specific activity within 1 min after tracer injection and that 90% of brain radioactivity at 1 min after injection is within stable lipids after passing through FA–CoA (see below) (6–8). Furthermore, tracer incorporation into stable brain lipids is independent of regional cerebral blood flow (rCBF), indicating that the model can be used for explicit imaging of incorporation (9). Flow independence reflects almost total (>99.99%) steady-state binding of plasma unacylated FA to plasma protein, a fast off-rate for its release from protein, its high permeability at blood-brain barrier, and rapid equilibration between unacylated FA in plasma and brain Fa–CoA (10–13). This can be seen as follows:

The extraction fraction E of a FA from blood by brain is defined as follows:

$$E = 1 - e^{-\alpha\tau}\,1 = 1 - e^{-aV/rCBF} \qquad [1]$$

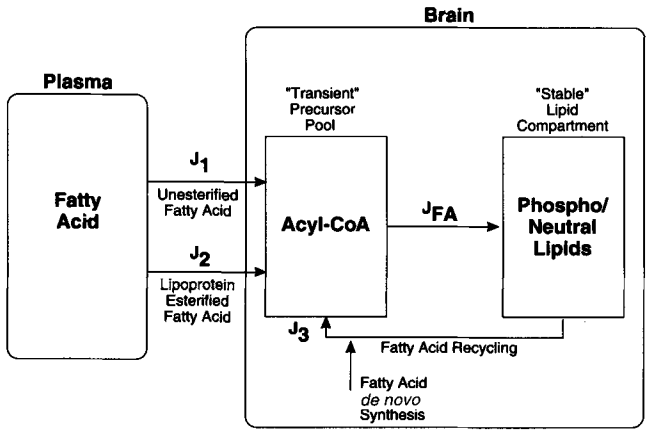


FIG. 1. Diagram for uptake of a fatty acid (FA) from plasma into brain FA (acyl)–CoA pool, and hence into stable phospholipids and neutral lipids. J_1, flux of unesterified FA from plasma into FA–CoA; J_2, flux of esterified FA on plasma lipoproteins into FA–CoA; J_3, flux from *de novo* synthesis and recycling from stable lipids into FA–CoA; J_{FA}, net flux of FA from FA–CoA to stable lipids. At steady state, $J_{FA} = J_1 + J_2 + J_3$

Abbreviations: FA, fatty acid; i.v., intravenous; NBM, nucleus basalis of Meynert; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; rCBF, regional cerebral blood flow

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where α is a rate constant (s^{-1}), τ is transit time of blood in brain (1 s in the rat), V is brain blood volume (cm^3 per g brain) and rCBF is in units of $cm^3.s^{-1}$ per g brain. Mean transit time $\tau = V/rCBF$ and as $E \approx 0.05$ (see above), Equation 1

$$E = \alpha\tau = \alpha V/rCBF \quad [2]$$

Brain uptake U ($\mu mol.s^{-1}$ per g brain) equals Ec_{bl}rCBF:

$$U = \alpha V c_{bl} \quad [3]$$

where c_{bl} (μmol per ml) is the arterial-end FA blood concentration. As capillary volume is independent of rCBF (14), uptake U also is flow-independent.

Brain Fa–CoA equilibrates rapidly with unacylated FA in plasma. Indeed, one min after a step elevation in plasma specific activity of [^{3}H]arachidonate (Fig. 2) or of [9,10-^{3}H]palmitate, specific activities of the respective unacylated FA and Fa–CoA pools in brain have reached steady states (7,8). Such rapid equilibration allows increased neuronal demand for incorporation of FA into phospholipids to be easily met by the large reservoir of unacylated FA in blood. As U is independent of rCBF, J_{FA} and J_l also will be flow-independent (6). Furthermore, incorporation of the FA into stable lipids from the Fa–CoA pool is so fast that 90% of brain radioactivity is acylated by 1 min after a radiolabeled-FA infusion (Fig. 3).

The net rate of incorporation of FA from Fa–CoA into brain phospholipid i (Fig. 1) is as follows (6):

$$J_{FA,i} = k^*_i c_{pl}/\lambda \quad [4]$$

where c_{pl} is cold plasma unacylated FA concentration and k^*_i ($cm^3.s^{-1}$ per g brain) is the incorporation coefficient of unacylated FA from plasma to phospholipid i. k^*_i equals brain radioactivity $c^*_{br,i}$ (T) in phospholipid i at time T of sampling, divided by the integral of plasma FA radioactivity from the beginning of infusion to time T (6):

$$k^*_i = c^*_{br,i} \div \int_0^T c^*_{pl}\,dt \quad [5]$$

λ is the extent to which Fa–CoA radioactivity due to labeled unacylated FA in plasma is diluted by unlabeled FA derived from acylated plasma sources and from brain recycling/*de novo* synthesis. At a steady state, λ is the ratio of specific activity of Fa–CoA in brain to specific activity of unacylated FA in plasma:

$$\lambda = \frac{J_1}{J_1 + J_2 + J_3} = \frac{J_1}{J_{FA}} \quad [6]$$

As $J_2 << J_1$ in adult rats (Purdon *et al.*, submitted for publication), a low value for λ would be caused by recycling and/or *de novo* synthesis.

At the steady state, turnover (%h) $F_{FA,i}$ of a FA in phospholipid i equals:

$$F_{FA,i} = \frac{J_{FA,i}}{c_{br,i}} = \frac{k^*_i c_{pl}}{\lambda c_{br,i}} \quad [7]$$

and thus, half-life (h) of FA in i equals:

$$t_{1/2,i} = 0.693/F_{FA,i} \quad [8]$$

Following an i.v. injection of a radiolabeled FA, maximum brain labeling occurs by 10 min and is mainly of lipids (>87%), particularly phospholipids (Tables 1,2). [9,10-^{3}H]palmitate labels mainly phosphatidylcholine (PC) in its *sn*-1 position, [1-^{14}C]arachidonate labels mainly phosphatidylinositol (PI) and PC in their *sn*-2 positions, and

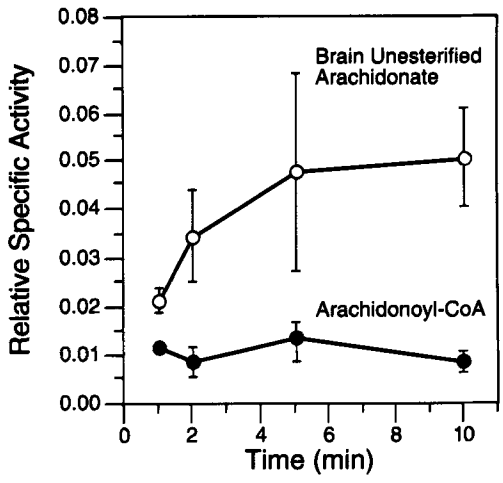


FIG. 2. Specific activity of arachidonate in brain unacylated arachidonate and arachidonoyl-CoA related to specific activity of unacylated arachidonate in arterial plasma of pentobarbital-anesthetized rats, following step elevation in plasma [^{3}H]arachidonate. Each point represents mean ± SEM for 3–4 animals, except for arachidonoyl-CoA at 1 min, when n = 1. From Washizaki *et al.* (7).

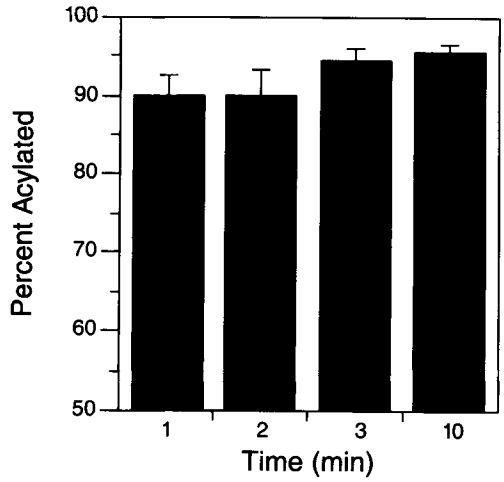


FIG. 3. Percent acylated [3H]arachidonate in brain of anesthetized rat, at various times after establishing a steady state plasma specific activity by i.v. infusion. Each bar represents mean + SD for 3-4 animals. From Washizaki *et al.*(7).

TABLE 1
Distribution of Radioactivity Within Brain Lipid Fractions in Awake Rats, 15 Min After a 5-min I.V. Infusion of Each of Three Labeled Fatty Acids[a]

Fraction	Fatty acid tracer (percent total brain radioactivity) [9,10-^{3}H]Palmitate	[1-^{14}C]Arachidonate	[1-^{14}C]Docosahexaenoate
Lipid fraction	89.2 ± 1.11	89.4 ± 0.49	87.1 ± 0.95
Phospholipids	51.7 ± 0.64	72.9 ± 0.80	64.7 ± 0.94
Sphingomyelin	1.21 ± 0.18	ND	ND
Phosphatidylcholine	31.7 ± 1.09	27.4 ± 0.22	17.2 ± 0.79
Phosphatidylserine	0.92 ± 0.20	2.09 ± 0.23	2.59 ± 0.30
Phosphatidylinositol	7.34 ± 0.20	35.6 ± 0.41	6.92 ± 0.68
Phosphatidylethanolamine	10.5 ± 0.94	7.14 ± 0.22	37.6 ± 0.82

[a]Values are mean ± SEM for 3–7 rats. From Nariai *et al.* (18) and Noronha *et al.* (15).

[1^{14}C]docosahexaenoate labels mainly phosphatidylethanolamine (PE) and PC in their *sn*-2 positions [Noronha, 190 #1501; Nariai, 1991 #1497]. Thus, combined use of the three FA tracers can provide estimates of *in vivo* half-lives and turnovers of specific acyl groups within specific brain phospholipids.

Half-lives of arachidonate and of palmitate within different brain phospholipids have been determined with Equations 7 and 8, using experimental values of λ (Table 3) (7,8). $t_{1/2,i}$ was shortest in PI of all the phospholipids for each of the FA, consistent with participation by PI in numerous activation processes (1,2). Furthermore, arachidonate had shorter half-lives within each phospholipid class than did palmitate, reflecting its involvement in phospholipase A_2-mediated signal transduction. The calculated $t_{1/2,i}$ of arachidonate in PC was 1.7 h (turnover = 41 %/h) compared with 9–14 d obtained with intracerebral injection and with 10–13 h in brain microsomes (4). Arachidonate-containing molecular species of PC and PI have been identified with half-lives <12 h (5), whereas our $t_{1/2,i}$ for arachidonate in PI was only 0.66 h (turnover = 105). Our calculated half-lives for palmitate ranged from 2.3 h (turnover = 30 %/h) to 13.3 h (turnover = 5.2%/h), much shorter than the published estimates of 3–23 d that ignored dilution (3).

A number of drugs can activate phospholipase A_2 via membrane-bound GTP-binding proteins, releasing arachidonate and other unsaturated acyl groups from *sn*-2 positions of phospholipids and thereby producing lysophospholipids (Fig. 4) (1). By prelabeling the Fa–CoA pool prior to drug administration by i.v. labeled arachidonate, for example, increased incorporation of label from the Fa–CoA pool into resultant lysophospholipids can be quantified and localized using autoradiography at sites of phospholipase A_2 activation. Thus, the muscarinic M_1 agonist arecoline in awake rats increased incorporation of injected [1-^{14}C]arachidonate into *sn*-2 positions of PI and PC, and of [1-^{14}C]docosahexaenoate into PE and PC, thereby increasing overall brain k* for each tracer (15) (Table 4). The effect on [1-^{14}C]arachidonate was blocked by atropine, and occurred in 83% of brain regions with M_1 receptors. In contrast, arecoline had no significant effect on incorporation of [9,10-^{3}H]palmitate, consistent with lack of involvement of *sn*-1 acyl groups on PC in cholinergic signal transduction.

Upregulation of phospholipase A_2-mediated signal transduction involving the M_1 receptor has been demonstrated using the FA method. The nucleus basalis of Meynert (NBM) provides cholinergic input to the ipsilateral neocortex and is pathological in humans with Alzheimer disease (16). Two weeks after a unilateral ablation of the NBM in rats, resting glucose metabolism and resting incorporation of [1-^{14}C]arachidonate have been reported to be bilaterally symmetrical (17,18), whereas arecoline administration increased incorporation of [1-^{14}C]arachidonate into the cortex on the lesioned side (Fig. 5) (18). In unstimulated rats, however, incorporation of [9,10-^{3}H]palmitate was increased into the ipsilateral cortex, consistent with evidence for membrane remodeling involving PC (De Micheli, E., unpublished data).

In this regard, incorporation of labeled palmitate into brain has been shown to be altered with a chronic lesion of the NBM (see above), Wallerian degeneration, chronic sensory

TABLE 2
Distribution of Radiolabeled Fatty Acids Within *sn*-1 and *sn*-2 Positions of Brain Phospholipids That Receive Most of the Label, 15 Min After I.V. Injection in Awake Rats[a]

Fatty acid tracers	Phospholipid	Percent distribution of radiolabel *sn*-1 position	*sn*-2 position
[9,10-^{3}H]Palmitate	PC	82.5 ± 1.9	17.5 ± 1.9
	PE	90.7 ± 0.5	9.3 ± 0.5
[1-^{14}C]Arachidonate	PC	7.8 ± 1.9	92.2 ± 1.9
	PI	0.0 ± 0.0	100 ± 0
[1-^{14}C]Docosahexaenoate	PC	22.9 ± 5.2	77.1 ± 5.2
	PE	0 ± 0	100 ± 0

[a]Values are mean ± SEM for 3–7 rats. From Nariai *et al.* (18).

TABLE 3
Half-Lives of Fatty Acids Within Individual Phospholipids of the Rat Cerebral Cortex[a]

Fatty acid	Phospholipid class PC	PS	PI	PE
	$t_{1/2,i}$, h			
Palmitate (16:0)	11.5	13.3	2.33	12.0
Arachidonate (20:4)	1.7	8.3	0.66	23.8

[a]Data calculated as described [Robinson, 1992 #1608] using λ = 0.011 for arachidonate, 0.0161 for palmitate (7,8). See Table 2 for abbreviations.

TABLE 4
Unidirectional Whole-Brain Incorporation Coefficients for Fatty Acid Tracers, Measured 15 Min Following 5-Min I.V. Infusion of Radiolabel and Intraperitoneal Administration of 15 mg.kg^{-1} Arecoline in Awake Rats

	Unidirectional Incorporation Coefficient, k*, cm^3.s^{-1} per g brain × 10^{-4}		
Fatty acid tracer	Control	Arecoline (15 mg/kg)	Change (%)
[9,10-^{3}H]Palmitate	0.88 ± 0.06	0.94 ± 0.04	+11
[1-^{14}C]Docosahexaenoate	3.08 ± 0.14	4.48 ± 0.26[a]	+48
[1-^{14}C]Arachidonate	3.13 ± 0.18	4.42 ± 0.27[a]	+41
[1-^{14}C]Arachidonate ± 4 mg/kg atropine[b]	3.30 ± 0.15	2.54 ± 0.31	−24

Mean ± SEM for 3–9 animals.
[a]Differs significantly from control mean, $P < 0.05$
[b]Atropine was given prior to arecoline infusion in one set of experiments, whereas methylatropine was administered routinely to prevent peripheral effects of arecoline. From DeGeorge *et al.* (15).

deprivation, recovery from transient ischemia and brain development, but not during acute changes in functional activity (15,18–24). These observations, and the fact that PC is the major phospholipid in cell membranes (25,26), suggest that labeled palmitate, which is incorporated into the *sn*-1 position of PC, can be used to examine neuroplasticity involving membrane remodeling or cell growth. [1-^{14}C]arachidonate and [1-^{14}C]docosahexaenoate appear to be more useful markers for acute changes in functional activity, and for chronic changes in phospholipase A_2-mediated signal transduction (see above) (15,24). In the future, [^{11}C]positron-emitting isotopes of long-chain FA might be used with positron emission tomography to examine human brain phospholipid metabolism *in vivo* in health and disease (27).

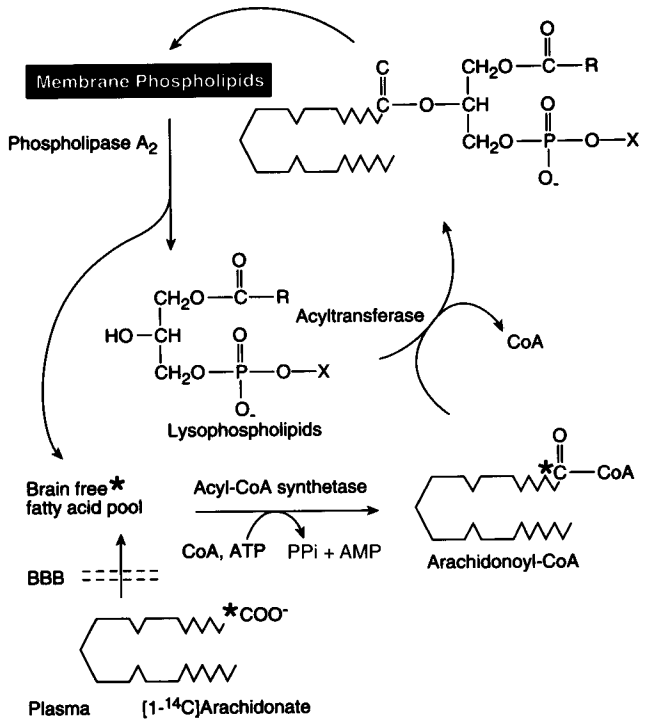


FIG. 4. Cycling of arachidonate following activation of phospholipase A_2. Unlabeled arachidonate that is released from a phospholipid (upper right) is reacylated into the brain acyl-CoA pool. The latter has been prelabeled by labeled unacylated [1-^{14}C]arachidonate in blood plasma, and provides labeled tracer for transacylation into resultant lysophospholipids.

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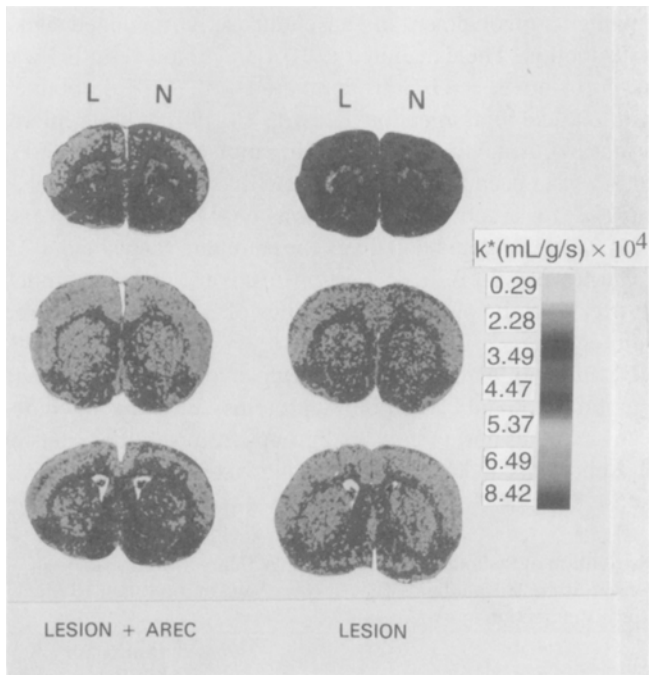


FIG. 5. Digitized color autoradiographs showing regional brain incorporation rates k^* of [1-^{14}C]arachidonate, two weeks after a unilateral lesion of the right nucleus basalis magnocellularis in adult rats. Animals received 170 mCi.kg^{-1} i.v. [1-^{14}C]arachidonate. Left column, brain of rat given 5 mg.kg^{-1} i.v. arecoline prior to FA tracer; right column, brain of control animal. L, lesioned side; N, nonlesioned side. Prefrontal and frontal cortices on lesioned side show increased tracer incorporation after arecoline, but no difference from nonlesioned side in animals not given drug. From Nariari *et al.* (18).

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Essential Fatty Acid Uptake and Metabolism in the Developing Rodent Brain

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ABSTRACT: Studies were carried out to determine whether the brain takes up and metabolizes essential fatty acids during early postnatal development in rodents. Rats and mice were dosed with deuterium-labeled linoleic and linolenic acids either by intraperitoneal injection or by gavage. Animals were killed at different times thereafter, and organs were removed. Brains, livers, and blood were analyzed by gas chromatography–negative-ion-mass spectrometry for labeled fatty acids. To determine whether fatty acids were present in the brain apart from cerebral blood, a subset of animals was exsanguinated by perfusion with buffered saline, and the brain was then fractionated into subcellular components. Results demonstrated that the brain took up both labeled essential fatty acids within 8 h from the time of dosing. There was on average a greater uptake of linolenic acid into the cerebellum than into the cerebral cortex during the first 8 d of life in rats. The amount of linoleic acid taken into either region was similar, however. Docosahexaenoic acid intermediates, 20:5n-3 and 22:5n-3, were also found labeled in the brain. Time-course labeling experiments indicated that these intermediates may be converted to 22:6n-3 within the brain. A rise of labeled 22:6n-3 in the brain at 24 h appeared to be due to uptake of this fatty acid from the blood. The amount of labeled 22:6n-3 in the brain continued to increase beyond 24 h, and this did not appear to be correlated with its blood concentration. These results suggest that, during development in the rodent, different regions within the brain may vary in their capacity to synthesize 22:6n-3, and this may be correlated with regional growth rates.
Lipids 31, S-103–S-107 (1996).

In mammals, there is rapid brain growth during prenatal and early postnatal periods of development, and accumulation of long-chain polyunsaturated fatty acids is important for overall neural tissue formation. In the rat, for example, growth of the cerebellum occurs almost entirely during the first 15 d after birth, whereas much of the cerebral cortex is formed before birth (1). In humans, the brain-growth period occurs from approximately the third trimester of pregnancy to about 18 months after birth (2). Over the past two decades, a number of published reports have suggested that the brain itself may be an alternative site of polyunsaturated fatty acid biosynthesis during development (2–12). One recent report in felines demonstrated that the brain rather than the liver produces neural 22:6n-3 from 22:5n-3 (13). Other investigators have suggested that the brain controls liver synthesis of docosahexaenoic acid and signals the liver to increase 22:6n-3 production when appropriate (14). In either case, experimental evidence is needed to clarify the brain's role in supplying neural long-chain polyunsaturated fatty acids during development to support either of these hypotheses.

The first report of the use of deuterium-labeled fatty acids to study fat metabolism was published in 1935 (15). It has only been through current advancements in modern chromatography techniques coupled with the application of the high sensitivity and specificity of mass spectrometry, however, that has led to the establishment of routine tools for trace analysis *in vivo* (16).

A study was carried out in rodents to determine whether the brain takes up and metabolizes 18-carbon essential fatty acids during the early postnatal period. Animals were dosed with deuterium-labeled fatty acids, and tissues were analyzed by gas chromatography–mass spectrometry for labeled metabolites. Regional accumulation of 18:2n-6 and 18:3n-3 was determined by dissecting the cerebellum away from the frontal cortex and analyzing the compartments separately. To accurately locate labeled fatty acids in the brain, 3 animals were perfused with a buffered saline solution to remove the blood. A comparison was made between liver, blood, and brain levels of d_5-22:6n-3 throughout 7 d following an oral dose of d_5-18:3n-3 ethyl ester.

EXPERIMENTAL PROCEDURES

All animal procedures were carried out in accordance with the National Institutes of Health (NIH) animal care and welfare guidelines, and the protocol was approved by the National Institute on Alcoholism and Alcohol Abuse (NIAAA) Animal Care and Use Committee. Mothers were maintained on a standard rodent diet, NIH-31, containing 22 energy-percent (en%) fat for the duration of these studies, and young animals suckled from their mothers. Maternal diet contained fatty acids in the following percentages:16:0 (3.4 en%), 16:1n-7 (0.5 en%), 18:0 (1.5 en%), 18:1n-9 (4.8 en%), 18:1n-7 (0.5 en%), 18:2n-6 (7.4 en%), 18:3n-3 (0.88 en%), 20:4n-6

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Abbreviations: en%, energy-percent; PFB, pentafluorobenzyl; SIM, selected ion chromatogram.

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(0.04%), 20:5n-3 (0.70 en%), and 22:6n-3 (0.55 en%). Deuterium-labeled fatty acid ethyl esters, d_5-18,18,18,17,17-18:2n-6 and d_5-18,18,18,17,17-18:3n-3, were obtained from Cambridge Isotope Laboratories (Woburn, MA).

Uptake of d_5-18:3n-3 and d_5-18:2n-6 in rat brain. Separate groups of rats on days 2, 3, 4, 5, 6, 7, and 8 of life were dosed with 1 mg of both d_5-18:2n-6 and d_5-18:3n-3 ethyl esters by gavage. Animals were killed by cervical dislocation 48 h after dosing, blood was taken, brains were removed, and the cerebellum and cortex were dissected out.

To determine if d_5-18:3n-3 was taken up into the brain as distinct from cerebral circulation, a group of three 2-day-old rats were dosed with 1 mg d_5-18:3n-3 ethyl ester, and 8 h later they were anaesthetized by placing them over wet ice. Chest cavities were opened, and animals were perfused with a buffered saline solution using a 33-gauge needle and syringe to puncture the right ventricles of the hearts. Brains were then removed and homogenized in phosphate buffered saline using a hand-held ground-glass homogenizing vessel. Homogenates were centrifuged at 12,000 × *g* for 20 min, producing the P1 pellet (whole cells and capillaries). The supernatant was then centrifuged at 100,000 × *g* for 1 h to obtain a microsomal pellet (P2).

Uptake of d_5-18:3n-3 and accumulation of d_5-22:6n-3 in mouse brain. Eighteen three-day-old C57BL/J6 mice were given an injection of 50 μg d_5-18:3n-3 directly into the intraperitoneal cavity using a 33-gauge needle. Animals were killed at 2, 4, 24, 48, 72, and 96 h after injection, the brain and liver were dissected out, and homogenized, and lipids were extracted.

Lipid extraction and pentafluorobenzyl (PFB) ester formation. Samples of homogenized brains (in some cases, cerebellum and cortex) and 100-mg pieces of liver were homogenized in water, and lipids were extracted using the Bligh and Dyer total lipid extraction procedure (17). Deuterium-labeled fatty acids were hydrolyzed and then derivatized to their pentafluorobenzl (PFB) esters using PFB bromide containing diisopropylethylamine in acetonitrile (1:10:1,000, by vol), as previously described (16).

Capillary gas chromatography–mass spectrometry analyses. Deuterium-labeled fatty acids were analyzed in the selected ion mode by gas chromatography–mass spectrometry on a Hewlett-Packard 5989 GC–MS (Palo Alto, CA), as previously described (18). Deuterium-labeled products were quantified using relative response factors for the individual analyses and an internal standard, 23:0, added in the nonesterified fatty acid form.

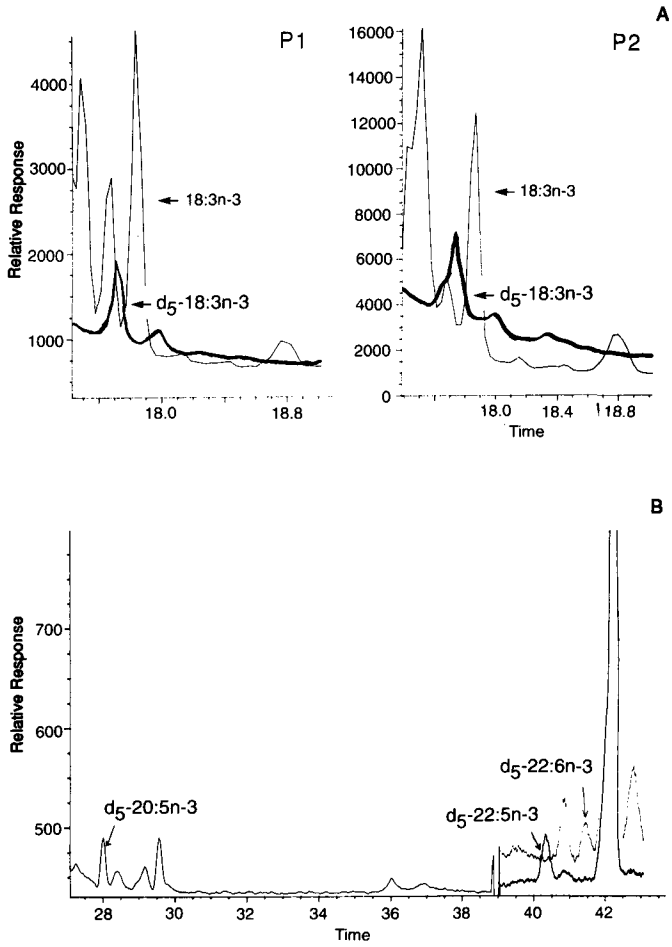


FIG. 1. A. Gas chromatography–mass spectrometry-selected ion chromatograms (SIM) for the ions *m/z* 277 and 282 of the fatty acyl pentafluorobenzyl (PFB) derivatives obtained from whole rat brain extracts. Animals were given a single oral dose of 1 mg d_5-18:3n-3 ethyl ester. The panel designated as P1 is the SIM tracing of the derivatized pellet obtained from low speed (12,000 × *g*) centrifugation of whole brain homogenate. The panel designated as P2 is the SIM chromatograph of the pelleted material resulting from the supernatant that was centrifuged at high speed (100,000 × *g*). The ion at *m/z* 277 represents the PFB ester of 18:3n-3, and the ion at *m/z* 282 represents d_5-18:3n-3. B). SIM chromatographs of the PFB derivatives of fatty acids extracted from whole rat brain homogenates. Chromatographs show the isotopically labeled fatty acids, 20:5n-3 at *m/z* 306, 22:5n-3, at *m/z* 334, and 22:6n-3 at *m/z* 332, 8 h after dosing animals with 1 mg d_5-18:3n-3 ethyl ester.

RESULTS

In the perfused whole rat brain, d_5-18:3n-3 was detected as early as 8 h after dosing (Fig. 1A). There was a 30% enrichment of the labeled 18:3n-3 in the microsomal fraction P2, as compared to a 16% enrichment in whole cells and capillaries, P1. This indicated that the isotope was associated with neural cells as well as being present in capillaries. The n-3 fatty acids, d_5-20:5n-3 and d_5-22:5n-3, were also detected in the rat brain (Fig. 1B). These results confirm other reports demonstrating that 18-carbon essential fatty acids are taken up into the neonatal brain, where they may be converted to long-chain polyunsaturated fatty acids (2–12).

In another experiment in which mice were dosed with d_5-18:3n-3 by intraperitoneal injection, labeled 18:3n-3 was observed in both brain and liver. In the liver after 24 h, the amount of d_5-18:3n-3 had decreased about eightfold from the maximum amount observed at 2 h. At the same time, the level of d_5-18:3n-3 had increased in the brain (Fig. 2). This contrasts with results reported by Scott and Bazan (14) where

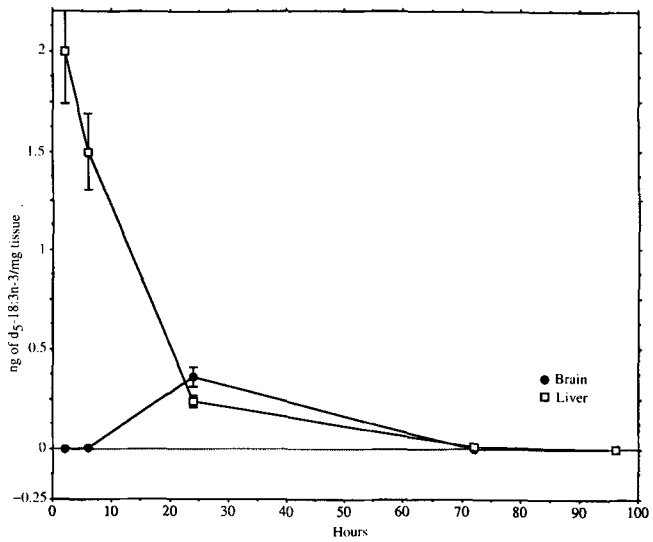


FIG. 2. Plots of the time course of d_5-18:3n-3 concentration in the livers and brains of 3-day-old mice (n = 3 for each time-point) given an intraperitoneal injection of 50 µg of d_5-18:3n-3. Livers and brains were sampled at 2, 6, 24, 72, and 96 h following the injection.

they failed to observe any uptake of labeled 18:3n-3 in the mouse brain following an injection of labeled 18:3n-3. The reason for these incongruous observations is unclear, but it may be related to differences in experimental methodology. The isotope labeling pattern of the fatty acids was determined in the earlier work using high-pressure liquid chromatography with radioactive detection. It is possible that this system did not have sufficient resolving power to separate the closely

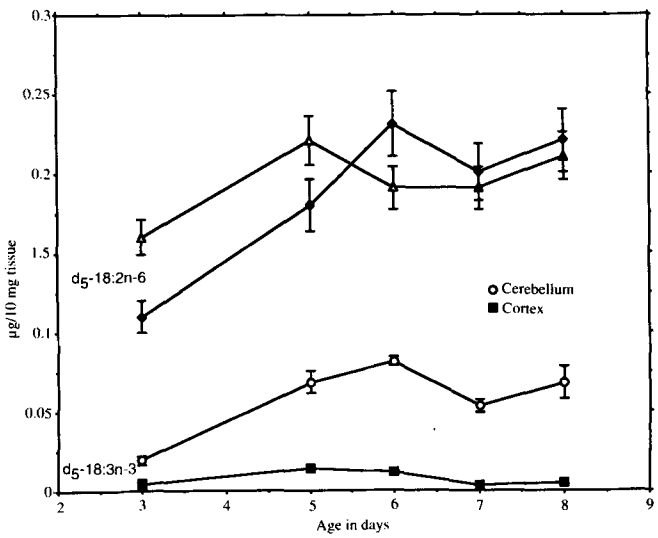


FIG. 3. Uptake of d_5-18:2n-6 and d_5-18:3n-3 into cerebellums and forebrains of neonatal rats (n = 3 for each time-point) after a 1-mg oral dose of the labeled fatty acids. Animals were killed 48 h after being given a dose of the isotope. The age indicates the time at which the sample was taken.

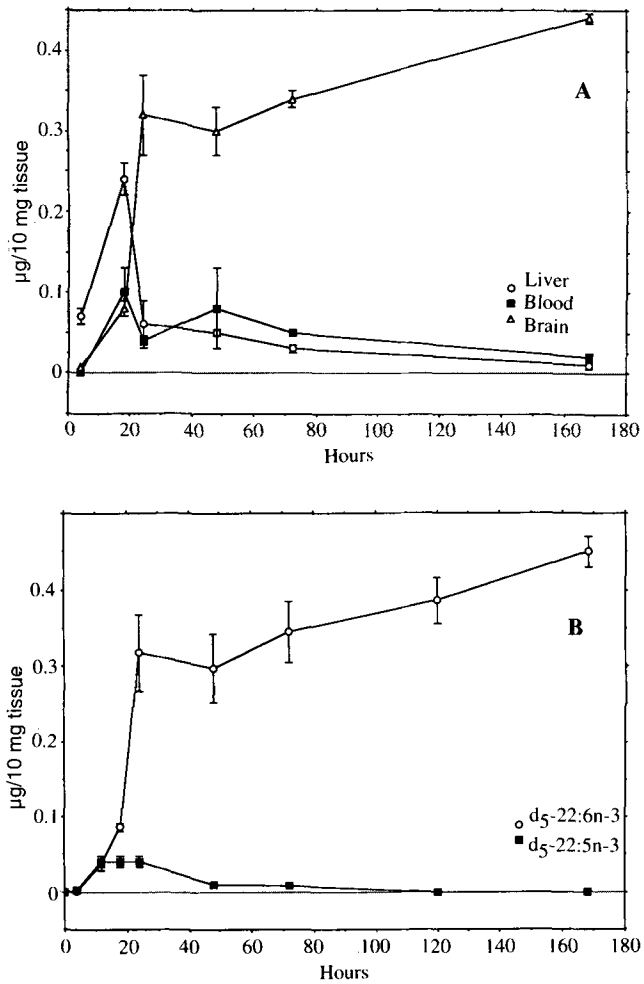


FIG. 4. A). The time course of d_5-22:6n-3 accumulation in the livers, blood, and brains of 3-day-old rats (n = 3 for each time-point) given a single oral dose of 1 mg d_5-18:3n-3 ethyl ester. B). The time course of d_5-22:6n-3 and d_5-22:5n-3 in the brains of 3-day-old rats (n = 3 for each time-point) that had been given a dose of 1 mg d_5-18:3n-3 ethyl ester.

eluting labeled 18:3:n-3 from 22:6n-3 (14). Also, there may be dietary differences in essential fatty acid intake between the two experiments.

In rats there was a much greater uptake of d_5-18:3n-3 into the cerebellum than into the cerebral cortex throughout the first 8 d of life (Fig. 3). There were similar amounts of d_5-18:2n-6 observed in both brain regions, however. This indicates that the developing cerebellum may have a greater requirement for n-3 fatty acids than does the cortex, and it suggests the possibility that different regions of the brain may vary in the level of docosahexaenoic acid synthesis. Alternatively, the higher level of d_5-18:3n-3 in the cerebellum may indicate that there is a lower conversion rate to d_5-22:6n-3 within that region.

In the rat brain it appeared that the primary accumulation of labeled docosahexaenoic acid resulted from liver production of 22:6n-3, which was subsequently taken up from the

blood (Fig. 4A). Isotope enrichment of 22:6n-3 reached a maximum in the liver and blood 18 h following the d_5-18:3n-3 dose; at 24 h there was a sharp increase in the amount of d_5-22:6n-3 in the brain. This concurs with the results reported by Scott and Bazan in mice (14). In the present study, however, isotopic enrichment of 22:6n-3 in the brain continued to increase after 24 h, while blood levels of d_5-22:6n-3 had fallen. At 168 h, there was a 25% greater enrichment of the 22:6n-3 in the brain than that at 24 h. One possible explanation for this increased enrichment is that other n-3 fatty acids are being converted to d_5-22:6n-3 within the brain. Previous studies have provided evidence for the brain conversion of n-3 fatty acids to 22:6n-3 (3–12). For example, appreciable amounts of labeled 20:5n-3 and 22:5n-3 were detected in brains of mice several hours after animals received a dose of labeled 18:3n-3 (14). These may have originated from the neural metabolism of labeled 18:3n-3. Furthermore, both labeled 20:5n-3 and 22:5n-3 decreased in the brain between 2 and 72 h. Moreover, when radiolabeled 18:3n-3 is injected into the brain, 22:6n-3 rapidly becomes labeled (4,5,12). In the present study, d_5-22:5n-3 decreased in the brain, while d_5-22:6n-3 increased (Fig. 4B).

DISCUSSION

Both neonatal mice and rats were found to take up labeled 18-carbon fatty acids into the brain. There was about a 15% lower amount of d_5-18:3n-3 in the perfused brain compared to nonperfused tissue, demonstrating that fatty acid was found in both the brain parenchyma and in cerebral arteries. Moreover, d_5-18:3n-3 was found in the P2 subcellular fraction of the brain. These results differ from an earlier report in mice (14) but confirm other reports in rats and other species (2–12). The diet used by Scott and Bazan (14) differed from the one used in this study, and this may partially account for these different observations.

In the rat during the first 8 d of life, the d_5-18:3n-3 was more highly enriched in the cerebellum than in the cerebral cortex. In contrast, the concentration of d_5-18:2n-6 was about the same in both compartments. This is significant in that, in the rat, development of the cerebellum, including the elaboration of dendrites and subsequent synapses, is occurring at a much greater velocity during this period than is the cortex (1). Therefore, increased accumulation of n-3 fatty acids may be a nutrient requirement for formation of these structures. Another possible explanation of this data is that there may be a lower conversion rate of 18:3n-3 to 22:6n-3 within the cerebellum, and this brings about a buildup of the precursor. This hypothesis, however, does not take into account that the cerebellum is growing at a rapid rate and presumably requires fatty acids for elaborating new membranes.

These findings also indicate that a portion of the labeled 22:6n-3 intermediates, such as d_5-20:5n-3 and d_5-22:5n-3 were metabolized to 22:6n-3 within the brain. It is significant that 18:3n-3 conversion to 22:6n-3 is occurring in both the liver and brain in neonatal animals when the maternal diet contains long-chain polyunsaturated fatty acids, since it is uncertain whether in young animals their synthesis is regulated by the inclusion of long-chain polyunsaturated fatty acids in the diet (18,19).

The present study supports the view that, in rodents during postnatal development, the liver contributes a major supply of 22:6n-3 to the brain for neural development. This study, however, also supports suggestions from previous work indicating that the developing rat brain has the capability to synthesize long-chain polyunsaturated fatty acids and that this may be an additional source of 22:6n-3 required during development (2–12). The findings suggest that, based on selected uptake of 18:3n-3, there may also be different regional requirements for and capabilities to biosynthesize 22:6n-3 in the brain. Based on these findings, the quantitative significance of brain biosynthesis of 22:6n-3 may be significant and suggest that more detailed studies of essential fatty acid metabolism and brain development are needed to fully understand the nature of this function in the brain.

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In Vivo Compartmental Metabolism of ^{13}C Docosahexaenoic Acid, Studied by Gas Chromatography-Combustion Isotope Ratio Mass Spectrometry

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ABSTRACT: The exchange of docosahexaenoic acid (22:6n-3) within lipid pools in rat and human has been followed as a function of time after the ingestion of triglycerides (TG) containing 22:6n-3 labeled with ^{13}C (^{13}C 22:6n-3). The ^{13}C abundance in the fatty acid was measured by gas-chromatography-combustion isotope ratio mass spectrometry which allowed the detection of 0.001 atom ^{13}C percent ^{12}C. The ^{13}C 22:6n-3 appearance was rapid in the TG of very low density lipoprotein plus chylomicron fraction, in which the maximal labeling was observed at 3 and 2 h after ingestion in rat and human, respectively. Concomitant with the TG utilization of this fraction by lipoprotein lipase from tissues, unesterified ^{13}C 22:6n-3 appeared in the plasma albumin. ^{13}C 22:6n-3 bound to albumin was mostly present in unesterified form before 12 h post-ingestion while after that period, lysophosphatidylcholine (lysoPC) bound to albumin carried higher ^{13}C 22:6n-3 concentrations. These lyso-PC were mostly from hepatic origin and might represent a potential source of 22:6n-3 redistribution to tissues. The ^{13}C 22:6n-3 uptake into rat brain PC and phosphatidylethanolamine was still increasing when the concentration of plasma unesterified ^{13}C 22:6n-3 had already dropped to a minimal plateau value and during the period of maximal plasma circulation of ^{13}C 22:6n-3-lysoPC bound to albumin. In contrast, the uptake of ^{13}C 22:6n-3 into blood platelet PC occurred during the phase of important circulation of ^{13}C-22:6n-3 bound to albumin, suggesting the *in vivo* efficiency of the Lands pathway for this fatty acid. It is concluded that ^{13}C 22:6n-3 esterified in TG is rapidly absorbed and redistributed within plasma lipoproteins and that its redistribution within the two lipid species bound to albumin might influence its uptake by platelets and rat brain.

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The n-3 family of polyunsaturated fatty acids has received much interest because of evidence that these compounds are antiatherogenic (1) and may also be essential nutrients (2). Among these fatty acids, docosahexaenoic acid (22:6n-3) is present in high concentrations in phospholipids (PL) of neurospecialized membranes from brain (3) and retina (4) and its depletion from these tissues leads to functional impairments in animals (5) and babies (6,7). The antiatherogenic properties of n-3 fatty acids result from their combined effects on blood lipids (8) and from their incorporation into cellular lipids where they interfere with the arachidonic acid cascade (1). It has been shown that the *in vitro* incorporation of 22:6n-3 into platelets decreased their aggregation and the binding of a thromboxane analogue to its receptor (9,10). The forms of transport of this fatty acid in plasma lipids for efficient distribution to blood cells and target tissues such as brain have been poorly reported.

In the present study, we have followed the 22:6n-3 exchange within lipid compartments, based on isotopic substitution, to establish precursor-product relationships in lipid synthesis. The metabolic fate of 22:6n-3 has been traced *in vivo* in rat and human using 22:6n-3 labeled by the stable isotope ^{13}C and a detection by gas chromatography-combustion isotopic ratio mass spectrometry (GCC-IRMS). Triglycerides containing ^{13}C 22:6n-3 (^{13}C 22:6-TG) were ingested by rats or human subjects and the appearance of the ^{13}C fatty acid was followed as a function of time in plasma lipoprotein, platelet and rat brain lipids.

It was shown that ^{13}C 22:6-TG was rapidly absorbed and redistributed within plasma lipoproteins and the albumin fraction and that the ^{13}C 22:6n-3 incorporation in lipid species bound to albumin influenced its uptake by platelets and rat brain.

METHODS

Synthesis of triglycerides containing [^{13}C]docosahexaenoic acid. These TG were produced by growing a microalgae strain in a defined medium containing 0.9 g/L (batch 1) or 3 g/L (batch 2) of D-[1-^{13}C] glucose (Eurisotop, Saint Aubin, France). In batch 1, ^{13}C 22:6n-3 represented 36 mol% of total TG fatty acids and had a ^{13}C abundance of 2.04 atom %. In batch 2, ^{13}C 22:6n-3 represented 46 mol% of total TG fatty acids and had a ^{13}C abundance of 7.72 atom %. Sixty % of the ^{13}C 22:6n-3 was located at the *sn*-2 position of the TG.

Rat experiment. Male Sprague-Dawley rats (300 g) were fed a standard lab chow diet (Souriffarat, Genthon,

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Abbreviations: GCC-IRMS, Gas chromatography-combustion isotope ratio mass spectrometry; HDL, high density lipoprotein; LDL, low density lipoprotein; LysoPC, lysophosphatidylcholine; PC, phosphatidylcholine; PDB, Pee Dee Belemnite; PE, phosphatidylethanolamine; PL, phospholipid; TG, triglyceride; VLDL, very low density lipoprotein.

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Cheyssieu, France). The tracer TG were taken to dryness under nitrogen and immediately dispersed into 600 µL of skim milk for rat intubation. After fasting the animals overnight, they received 3 mg of ^{13}C 22:6-TG from batch 1 or 2, by intragastric intubation and were further left free access to a solution of water containing 2% glucose, 0.5% NaCl and 0.01% KCl (wt/vol) until sacrifice. At various times after feeding, animals were anaesthetized by peritoneal injection of pentobarbital and 10 mL of blood were taken via aortic puncture in chilled plastic syringes containing acid-citrate-dextrose as anticoagulant (0.8% citric acid, 2% citrate, 2.45% dextrose, pH 4.5).

Human experiment. Overnight fasted healthy subjects absorbed the tracer TG (280 mg of batch 2) mixed in a yogurt and had breakfast immediately after this. At various periods of time after ingestion, blood was taken by venopuncture into chilled plastic syringes containing acid-citrate-dextrose. Blood sample was also collected just before the tracer ingestion to measure the basal ^{13}C abundance in lipid pools.

Preparation of biological samples. All experimental steps were performed at 4°C. Blood samples were immediately centrifuged at 100 g for 15 min, to obtain platelet-rich plasma (PRP). PRP was centrifuged at 1000 g for 10 min and the supernatant was treated for lipoprotein preparation. The resulting platelet pellet was rinsed twice and gently resuspended into a Tyrode/Hepes buffer, containing 1 mM EDTA, pH 6.4 (11). This cell suspension was immediately centrifuged at 1000 g for 10 min, rediluted into Tyrode/Hepes buffer, without EDTA pH 7.35, and frozen at −20°C. Lipoproteins were separated into very low density lipoproteins (VLDL) plus chylomicrons (VLDL/Chyl), low density lipoproteins (LDL), high density lipoproteins (HDL) and a nonlipoprotein fraction containing albumin by ultracentrifugation at 600,000 g in a fixed angle rotor (Kontron, TFT 80.49) for 2 h at 4°C, using NaCl/KBr density gradient (11).

Fractionation and quantification of lipids. Total lipids were extracted from plasma, platelets, and lipoprotein preparations according to Bligh and Dyer (12). The extraction of brain lipids was performed according to Folch (13). Lipid classes were prepared by spotting the lipid extract on silica gel 60 plates and eluting the total PL, free fatty acids, TG and sterol esters (SE) with hexane/diethyl ether/acetic acid (80:20:1, by vol), as a mobile phase. Lipids from lipoproteins and the albumin fraction were separated into lysophosphatidylcholine (lysoPC), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) on thin-layer chromatography plates with chloroform/methanol/H_2O (65:25:4, by vol), as the mobile phase. Platelet, red blood cell, and brain PL classes were purified by TLC migration with chloroform/methanol/14% aqueous methylamine (60:20:5, by vol) as a mobile phase, after predeveloping the plate with chloroform/methanol (80:8, vol/vol). Lipid classes were scraped off the plate and treated with 5% H_2SO_4 in methanol for 90 min for fatty acid methyl ester preparation. Lipid classes were quantified by adding internal standards in the extraction mixture. Triheptadecanoylglycerol, 1,2-diheptanoyl-glycero-3-phosphocholine and -phosphoethanolamine, heptadecanoyl-lysoPC and heptadecanoic acid (17:0) (Sigma Chemical, St. Louis, MO) were added when appropriate and the absolute amount of fatty acid methyl esters were determined by gas chromatography (GC) analyses, relative to the known amount of added 17:0. Fatty acid methyl esters were analyzed and quantified by GC, using an SP 2380 capillary column (30 m × 0.32 mm; Supelco, Bellefonte, PA). Prior to their GCC-IRMS analysis, fatty acid methyl esters were purified by HPLC using a 5 µm Superspher 100 C18 column (Merck, Darmstadt, Germany) and an isocratic elution with acetonitrile/H_2O. They were detected by UV absorbance at 210 nm, collected and the different fractions were rapidly taken to dryness under nitrogen.

GCC-IRMS analyses. Samples were injected into an HP 5890 gas chromatograph equipped with a Ross injector and a Supelco capillary column (SP-2380, 0.25 mm × 30 m). A heart-cut valve directed the helium flow either to the flame ionization detector or to a quartz tube filled up with CuO, heated at 800°C, where CO_2 was generated from fatty acids by catalytic combustion. The effluent was then driven into a water trap before its ionization by electron impact in the source of the isotope ratio mass spectrometer (SIRA 12; VG Isogas, Middlewich, United Kingdom). The different isotopomers were collected onto three different collectors at *m/z* 44, 45, and 46. Before and after the CO_2 peaks generated from the fatty acids, a CO_2 sample reference of known enrichment calibrated against the international standard (Pee Dee Belemnite: PDB) was automatically injected into the mass spectrometer (11).

Expression of results. $^{13}C/^{12}C$ of samples and reference were used to calculate the δ per 1000 value ($\delta^{13}C‰$) with the following equation:

$$\delta^{13}C‰ = \frac{(^{13}C/^{12}C \text{ sample} - {}^{13}C/^{12}C \text{ reference}}{{}^{13}C/^{12}C \text{ reference})} \times 10^3 \quad [1]$$

where the reference is the international PDB standard, which is CO_2 obtained from the carbonate shell of a cretaceous mollusk. As this international standard is rich in ^{13}C, the natural ^{13}C abundance of samples are negative $\delta^{13}C‰$ values. Results were also expressed as ^{13}C atom percentage (atom %). The atom % excess could be calculated by subtracting the basal ^{13}C abundance from the ^{13}C abundance in samples. The ^{13}C 22:6n-3 appearance in biological samples was also expressed as the absolute amount of ^{13}C 22:6n-3 in the lipid pools at various time points by determination of ^{13}C 22:6n-3 dilution in the pools with a standard curve and by multiplying this value by the endogenous 22:6n-3 concentration, measured by GC.

RESULTS

Rat experiment. The ^{13}C 22:6n-3 appearance in VLDL/Chyl-TG was followed as a function of time after the ingestion of the ^{13}C 22:6-TG labeled at 2.04 atom % (Fig. 1). The labeling in this fraction increased from basal $\delta^{13}C‰$ values to

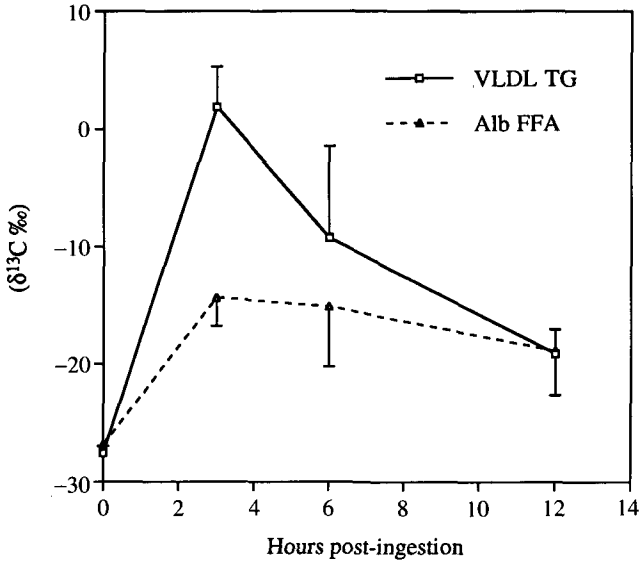


FIG. 1. The appearance of ^{13}C 22:6n-3 in very low density lipoprotein/chylomicron-triglycerides (VLDL-TG) and in unesterified fatty acids bound to albumin (Alb FFA), as a function of time after the ingestion of ^{13}C 22:6n-3-triglycerides. Rats were given a 3 mg intragastric dose of ^{13}C 22:6n-3-triglycerides labeled at 2.04 atom %. Lipoprotein and albumin fractions were prepared by ultracentrifugation at 600,000 g for 2 h and the lipid classes were purified by thin-layer chromatography. Each point represents the mean ± SEM of 3–4 rats.

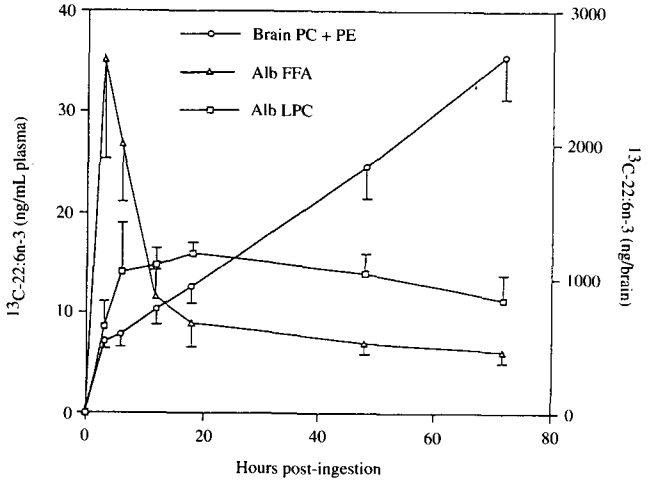


FIG. 2. The time course of ^{13}C 22:6n-3 concentrations in rat plasma unesterified fatty acids bound to albumin (Alb FFA), in plasma lysophosphatidylcholine bound to albumin (Alb LPC) and in brain phosphatidylcholine plus phosphatidylethanolamine (Brain PC + PE), after the ingestion of the ^{13}C 22:6n-3-triglycerides labeled at 7.72 atom %. The ^{13}C 22:6n-3 amount in each lipid class was calculated from the δ^{13}C‰ values and from the 22:6n-3 endogenous concentrations. Each point represents the mean ± SEM of 3–5 rats.

+1.87 ‰, 3 h after ingestion and was already maximal at this time point to further decline until 12 h. The ^{13}C abundance in 22:6n-3, from unesterified fatty acids bound to albumin, paralleled the evolution observed in VLDL/Chyl-TG with a maximal appearance of −14.39‰ at 3 h and further declined. In rats having absorbed the ^{13}C 22:6-TG labeled at 7.72 atom %, the ^{13}C 22:6n-3 appearance was followed in the plasma albumin. Two lipid species carrying significant amounts of endogenous 22:6n-3 were identified in this fraction. The unesterified fatty acids and lysoPC contained 1.35 ± 0.34 μg/mL and 1.55 ± 0.38 μg/mL of endogenous 22:6n-3, respectively (mean ± SD). The time course of ^{13}C 22:6n-3 appearance was different in the two lipid species. A maximal δ^{13}C‰ value of +189‰ was observed in unesterified fatty acids 3 h after ingestion followed by a decline of the labeling until 12–18 h and a plateau from these time points to 72 h post-ingestion (not shown). In contrast, the labeling of lysoPC was more progressive until 6 h and plateaued from 6 to 72 h to a δ^{13}C‰ value which was around 4-fold higher in lysoPC than in unesterified fatty acids (not shown). The ^{13}C 22:6n-3 appearance in these two lipid species was also expressed as the absolute amount of ^{13}C 22:6n-3 at various time points (Fig. 2). While the ^{13}C 22:6n-3 concentration was higher in unesterified fatty acids than in lysoPC at 3 h, it reached comparable levels at 12 h and plateaued until 72 h post-ingestion where it was twice higher in lysoPC than in unesterified fatty acids. The ^{13}C 22:6n-3 appearance into brain PL was followed as a function of time after the tracer ingestion. The magnitude of isotopic variations was moderate in PC and PE, due to the huge dilution of the tracer in the endogenous brain 22:6n-3. The sum of ^{13}C 22:6n-3 incorporation in PC and PE was reported in Figure 2. It increased progressively over all the periods studied and did not plateau after 12 h during the phase of minimal plasma circulation of ^{13}C 22:6n-3 bound to albumin.

Human experiment. The ^{13}C 22:6n-3 appearance in TG from VLDL/Chyl, HDL and LDL in a healthy human subject was followed as a function of time after the ingestion of 280 mg of the tracer TG labeled at 7.72 atom % (Fig. 3A). The δ^{13}C‰ value was maximal in VLDL/Chyl-TG at 2 h which was the earliest time point investigated, dropped until 24 h and then plateaued throughout the end-period. A concomitant maximum of labeling at 3 h was observed in LDL-TG with a lower δ^{13}C‰ value than in VLDL/Chyl-TG (+1100 ‰ vs. +3500 ‰, respectively). The labeling increased in HDL-TG until 6 h where it reached +1400 ‰ and declined to a plateau value similar to the two other TG fractions (+110 to +120 ‰ at 72 h). The labeling in VLDL/Chyl-PL was higher than in HDL- and LDL-PC, until 24 h, where it reached comparable plateau values. The labeling was the highest in PE from LDL and HDL, but it decreased after 24 h in both fractions (Fig. 3 B,C). A comparison of the ^{13}C 22:6n-3 appearance in HDL-PC and lysoPC bound to albumin in three subjects having absorbed the tracer labeled at 7.72 atom % is reported in Figure 4. The labeling was always higher in lysoPC than in HDL-PC, refuting a precursor-product relationship from HDL-PC to lysoPC by the action of lecithin cholesterol acyl transferase (LCAT) in plasma. The ingestion of the tracer induced a sharp and rapid rise in platelet PC, followed roughly by a plateau

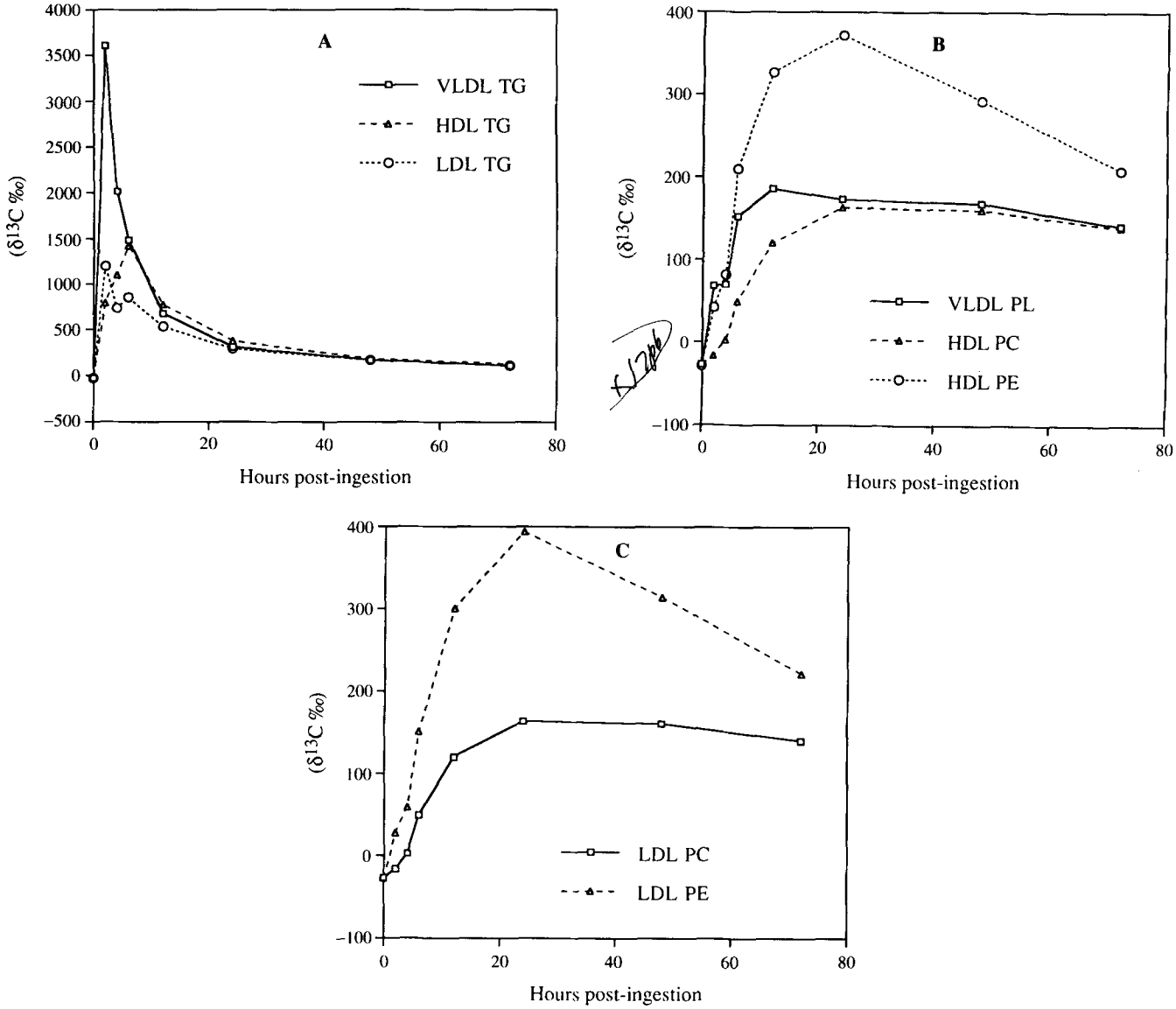


FIG. 3. Time course for the appearance of ^{13}C 22:6n-3 in lipoproteins from a human subject after a single ingestion of the ^{13}C 22:6n-3-triglycerides labeled at 7.72 atom %. (A) in triglycerides from VLDL plus chylomicrons (VLDL-TG), from high density lipoprotein (HDL) (HDL-TG) and from low density lipoprotein (LDL) (LDL-TG). (B) in phospholipids from VLDL (VLDL-PL), in PC and PE from HDL (HDL-PC, HDL-PE). (C) in PC and PE from LDL (LDL-PC, LDL-PE). Abbreviations as in Figures 1 and 2.

over the 12–48 h period (Fig. 5). The comparison with the kinetics of ^{13}C 22:6n-3 bound to albumin shows that ^{13}C 22:6n-3 accumulated into platelet PC during the phase of maximal circulation in plasma of the ^{13}C 22:6n-3 bound to albumin. Finally, since the tracer TG ingested had no detectable ^{13}C 22:5n-3 and ^{13}C 20:5n-3, the retroconversion of ^{13}C 22:6n-3 in rat and human was calculated by summing the amounts of ^{13}C 22:5n-3 and ^{13}C 20:5n-3 measured in plasma PL, TG, and SE 3 h after the ingestion of the tracer (batch 2) (Table 1). The retroconversion of ^{13}C 22:6n-3 appeared to be 8-fold higher in rat than in human.

DISCUSSION

Utilization of stable isotopes for biomedical research has been stimulated by the concern in using radiotracers in humans and by the recent development of sensitive techniques for quantitative detection of stable isotopes (14). Results of the present study show the potential of GCC-IRMS to trace ^{13}C 22:6n-3 in lipid compartments *in vivo*, even after a dose of ^{13}C fatty acid, only double of the natural abundance. Precision of the measurements is dependent on accurate determinations of the baseline due to natural ^{13}C enrichment. The baseline ^{13}C content in 22:6n-3 from four control rat lipid classes was 1.0830 ± 0.0005 atom %. This ensures precise determinations of very low ^{13}C abundance after tracer intake, as observed in tissues presenting poor uptake and/or high labeling dilution. For example, an uptake of 0.00086 ± 0.00018 atom % excess could be detected in rat brain PE 3 h after ingestion of the tracer labeled at 7.72 atom %. Moreover, the $^{13}C/^{12}C$ ratio, the equivalent of specific activity of radioisotopes, is direct isotopic

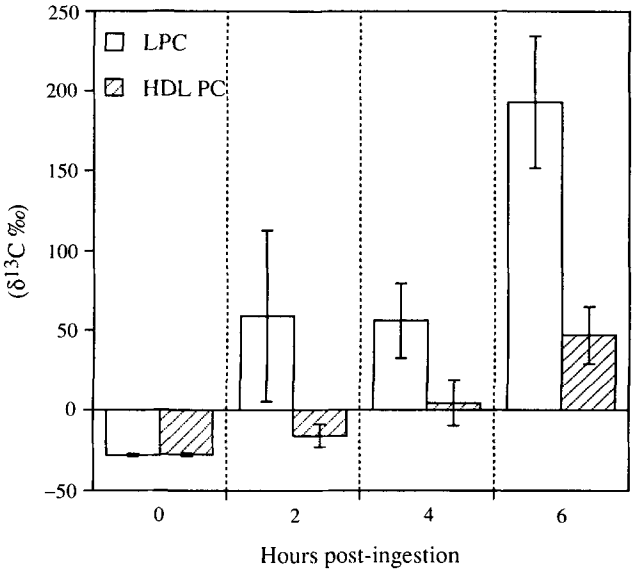


FIG. 4. The ^{13}C 22:6n-3 appearance in lysophosphatidylcholine bound to albumin (LPC) and in phosphatidylcholine from HDL (HDL-PC) in human subjects having absorbed the ^{13}C 22:6n-3-triglycerides labeled at 7.72 atom %. Results are the mean ± SD from three subjects. Abbreviations as in Figure 3.

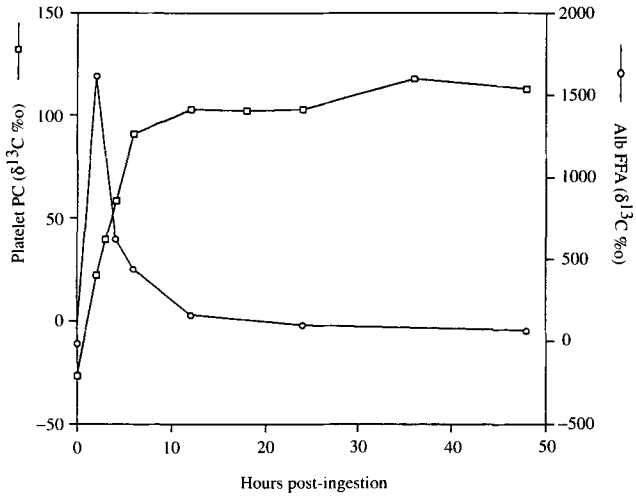


FIG. 5. A comparison of the ^{13}C 22:6n-3 appearance in platelet phosphatidylcholine (Platelet PC) and in plasma unesterified fatty acids bound to albumin (Alb FFA) in a human subject after a single ingestion of the ^{13}C 22:6n-3-triglycerides labeled at 7.72 atom %.

spectrometer data, independent of recovery and laboratory treatments.

^{13}C 22:6n-3 esterified in the tracer TG was rapidly absorbed in view of the maximal appearance of ^{13}C 22:6n-3 in VLDL/Chyl-TG at 2 and 3 h which were the earliest time points of kinetics, in human and rat, respectively. Concomitant with the utilization of the VLDL/Chyl-TG by lipoprotein lipase from tissues using this fatty acid for their own lipid synthesis, unesterified ^{13}C 22:6n-3 appeared in the plasma albumin fraction. Albumin is the known carrier of unesterified fatty acids in plasma mammals (15). Confirming a previous study in rat, where ^{14}C 22:6n-3 was given as free fatty acid by gavage (16), the present work shows that albumin also carries 22:6n-3 in rat and human after ingestion of the fatty acid esterified in TG, which is its natural form of absorption. The quantification of the ^{13}C 22:6n-3 bound to albumin, done in rats having absorbed the tracer labeled at 7.72 atom %, shows that before 12 h post-ingestion, ^{13}C 22:6n-3 was mostly present in unesterified form, but by 12 h, lyso-PC transported higher concentration of ^{13}C 22:6n-3. It has been shown that albumin stimulates the release of lyso-PC from isolated perfused rat liver and cultured hepatocytes (17). These hepatic lysoPC are mostly unsaturated which is different from the saturated lysoPC synthesized by LCAT in plasma. A physiological function of the lysoPC release from the liver seems to be the supply of extrahepatic tissues with choline and unsaturated fatty acids (18). The present study shows a quantitatively important synthesis of ^{13}C 22:6n-3-lysoPC *in vivo*, which could be a potential source of 22:6n-3 redistribution to tissues. It is generally admitted that the brain, which appears to contain little lipoprotein lipase, does not absorb TG readily, but that the classical route for its fatty acid supply would be the fatty acids bound to albumin (19). However, Anderson *et al.* (20) has recently reported that the developing rat brain can incorporate chylomicron fatty acids; other possible forms of supply, such as lyso-PC, have also been suggested (18,21). Our time course shows that the brain ^{13}C 22:6n-3 uptake into PC and PE was not maximal at 12 h when the labeling in plasma VLDL/Chyl-TG and unesterified fatty acids had already dropped to a minimal plateau value. The uptake linearly increased until three days after ingestion, suggesting other supply forms, such as ^{13}C-22:6n-3-lyso-PC, the concentration of which plateauing after 6 h. These observations are supported by recent work reporting that ^{3}H-22:6n-3 brain uptake was higher in young rats injected with ^{3}H-22:6n-3-lyso-PC than with unesterified 3H-22:6n-3 (22).

In humans, the rapid ^{13}C 22:6n-3 incorporation into VLDL/Chyl-TG was accompanied by the concomitant appearance of ^{13}C 22:6n-3 in LDL-TG, with a maximal δ^{13}C‰ value at 2 h. The maximal δ^{13}C‰ values of VLDL/Chyl-TG

TABLE 1
The Retroconversion of ^{13}C 22:6n-3 in Rat and Human[a]

	Rat (ng/mL plasma)		Human (ng/mL plasma)	
^{13}C 22:6n-3	1002 ± 192		1190 ± 651	
^{13}C 22:5n-3	17.1 ± 4.4	(1.7%)	6.92 ± 3.18	(0.58%)
^{13}C 20:5n-3	69.6 ± 15.2	(6.9%)	4.69 ± 4.98	(0.39%)
		(8.6%)		(0.97%)

[a]The retroconversion of ^{13}C 22:6n-3 was calculated by summing the amounts of ^{13}C 22:5n-3 and ^{13}C 20:5n-3 measured in plasma phospholipids, triglycerides, and sterol esters, 3 h after the ingestion of the tracer. Numbers in brackets represent the percentage of ^{13}C 22:6n-3 metabolized in ^{13}C 22:5n-3 or ^{13}C 20:5n-3. Results are the mean ± SEM of 3–4 rats and the means ± SD of three human subjects.

and LDL-TG was compatible with the precursor-product relationship between these fractions and shows the rapid synthesis of LDL from VLDL by degradation of TG and some PL (23). The maximal $\delta^{13}C‰$ value in HDL-TG was observed at 6 h, indicating that HDL progressively took up TG, probably by combined mechanisms of cholesteryl ester transfer protein activity and lipolysis of TG-rich lipoproteins (24). The ^{13}C 22:6n-3 appearance at the beginning of the time course was more rapid in VLDL/Chyl-PL than in PL from HDL or LDL, in accordance with their known secreted origin from liver and intestine (23). The ^{13}C 22:6n-3 appearance was higher in HDL- and LDL-PE than in the corresponding PC and this might result from a preferential affinity of hepatic ethanolaminephosphotransferase for 1-saturated,2-docosahexaenoyl precursors (25). The decrease of ^{13}C 22:6n-3 appearance observed in HDL- and LDL-PE, at around 18 h might be explained by the hepatic N-methylation of PE which is preferential for 22:6n-3-PE (26) or by the activity of hepatic lipase using PE as a specific substrate (27).^{13}C-22:6n-3-lysoPC bound to albumin was also synthesized, as observed in rat, quickly after ingestion and the $\delta^{13}C‰$ values were always higher than in the corresponding HDL-PC. This excludes a possible origin of ^{13}C 22:6n-3-lysoPC as product of plasma LCAT activity on the saturated fatty acids of HDL-PC and shows that ^{13}C 22:6n-3-lysoPC is mostly synthesized from a highly enriched precursor. The origin of ^{13}C 22:6n-3-lysoPC as liver secretory product is well in accordance with the previous observation that lysoPC produced by perfusion of rat livers is predominantly unsaturated (17).

The appearance of ^{13}C 22:6n-3 in platelet PC was rapid and reached the maximal plateau $\delta^{13}C‰$ values at around 6 h after the tracer ingestion during the phase of maximal plasma circulation of unesterified ^{13}C 22:6n-3 bound to albumin. It is known that blood platelets exchange *in vitro* their PL fatty acids with medium fatty acids bound to albumin by a acylation/deacylation pathway, the Lands pathway, although the importance of this metabolism has not been proved *in vivo* (28). The present observation shows that platelets continuously exchange their PC fatty acids with plasma fatty acids during their circulating life span and proves that 22:6n-3 is a substrate for the Lands pathway *in vivo*.

Finally, the quantification of ^{13}C 22:5n-3 and ^{13}C 20:5n-3 formed by retroconversion at 3 h, time point of maximal appearance of these fatty acids, showed that the tracer circulated mostly as ^{13}C 22:6n-3, although the retroconversion was higher in rat than in human. Whether the latter difference was due to species specificity or resulted from the endogenous amounts of 22:6n-3 in plasma lipids, which is higher in rat than in human, remains to be determined.

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Synthesis of Fat in Response to Alterations in Diet: Insights from New Stable Isotope Methodologies

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ABSTRACT: Synthesis of fatty acids, or *de novo* lipogenesis (DNL), is an intensively researched metabolic pathway whose functional significance and metabolic role have nevertheless remained uncertain. Methodologic problems that limited previous investigations of DNL *in vivo* and recent methodologic advances that address these problems are discussed here. In particular, deuterated water incorporation and mass isotopomer distribution analysis techniques are described. Recent experimental results in humans based on these techniques are reviewed, emphasizing dietary and hormonal factors that modulate DNL and quantitative significance of DNL under various conditions, including carbohydrate overfeeding. The somewhat surprising finding that DNL appears not to be a quantitatively major pathway even under conditions of surplus carbohydrate energy intake, at least in normal adults on typical Western diets, is discussed in depth. Nutritional and metabolic implications of these results are also noted, and some speculations on possible functional roles of DNL in normal physiology and disease states are presented in this context. In summary, methodologic advances have added to our understanding of DNL and its regulation, but many questions concerning quantitation and function remain unanswered.

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The enzymatic machinery for endogenous synthesis of fatty acids (FA) from acetate subunits is present in microbes, plants, and animals. Many biochemists have devoted their careers to studying genes and proteins involved in this pathway, also termed *de novo* lipogenesis (DNL). Nevertheless, a central functional question has remained unanswered: How much DNL actually occurs in organisms that can also ingest exogenous fats? A widely held concept is that DNL serves as a disposal route for surplus nonfat calories, especially carbohydrates (CHO) and ethanol, into the storage pool of body fat. This model of DNL as an overflow valve within the cellular or organismal energy economy has been difficult to evaluate because reliable techniques for measuring true rates and metabolic sources of DNL in living organisms until recently had not been available. Over the past three or four years, however, two methods have been developed that allow direct and accurate measurement of DNL in humans: mass isotopomer distribution analysis (MIDA) and deuterated water incorporation. A somewhat surprising experimental result has emerged, based on these methods—namely, that DNL is not a quantitatively important pathway under most circumstances in humans. These findings make the functions of DNL less certain, but potentially more interesting.

Here I review recent advances related to measurement and physiologic roles of DNL. First, I discuss the central methodologic problem that had to be resolved for measurement of DNL with isotopes (labeling of the intracellular true precursor pool) and how MIDA and ^2H_2O incorporation resolved this problem. Then I describe recent investigations using MIDA to measure DNL in humans under a variety of conditions, and I conclude with a discussion of the possible functions of DNL.

Overview of DNL as a pathway. Several features of DNL must be noted when considering the synthesis of FA as an integrated pathway rather than as a series of component enzymes. The first point is the thermogenic cost. Conversion of CHO to FA prior to oxidation dissipates an estimated 28% of the energy available to the organism if the carbohydrates were oxidized directly (1). This high thermogenic cost has variously been considered a reason for the organism to avoid DNL (energy wasteful) or a useful mechanism of thermogenesis (e.g., in brown fat, or to prevent weight gain during CHO overfeeding). The second point is that true DNL implies synthesis from 2-carbon units and should be distinguished from elongation of preformed FA. Though both DNL and elongation involve addition of activated acetate units in the form of malonyl-CoA, the former process produces nonessential FA, by definition, while the latter interacts with essential FA as well. The third aspect to consider is the current enzymologic model of the regulation of DNL. The malonyl-CoA/acetyl-CoA carboxylase (ACC) regulation model of DNL (2–7) is one of the most powerful and unifying of contemporary meta-

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Abbreviations: ACC, acetyl-CoA carboxylase; AIDS, acquired immune deficiency syndrome; CHO, carbohydrates; CS, cigarette smoking; DNL, *de novo* lipogenesis; FA, fatty acids; HGP, hepatic glucose production; MIDA; mass isotopomer distribution analysis; NIDDM, noninsulin-dependent diabetes mellitus; RQ, respiratory quotient; TG, triacylglycerols; VLDL, very low density lipoprotein.

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bolic hypotheses. The model is that malonyl-CoA concentrations integrate FA oxidation and synthesis (2,3) and prevent concurrent activity of these opposing pathways. Furthermore, malonyl-CoA concentrations are in turn sensitive to CHO-derived metabolites in the cell (oxaloacetate, pyruvate, citrate) and to the activity of the hormonally controlled enzyme ACC (4–7). In this biochemical context, the fourth point is the presumed function of DNL in the cellular and organismal energy economy. Based on its obvious function in plants and single-cell organisms as a route for storage as lipids of excess non-lipid-derived energy, the same function for DNL has been presumed for omnivorous mammals such as humans. Enzymology and molecular genetics of regulation have supported this view in that factors relating to CHO availability (substrates and hormones) regulate activity and synthesis of the enzymes of DNL (8–10). As we see later in the paper, however, quantitative investigations have not supported this as the usual physiologic function of DNL, at least in adults with typical Western diets. Finally, tissue sites of DNL should be noted. Species differences exist regarding relative importance of liver vs. adipose tissue as the major site of DNL. In rodents, adipose DNL may be at least as important as hepatic DNL (11), whereas for humans the general consensus is that liver is the predominant site (12,13). This formulation may not always be correct for humans, however. In addition, other specialized tissues may also perform DNL under certain circumstances, e.g., lactating mammary gland (14).

Measurement of DNL: historical problems. It may seem peculiar that some of the questions just mentioned remain unanswered. Why don't we know more definitively the quantitative importance of DNL, or the tissue site of DNL in humans, or the relation between molecular genetic and physiologic events? The main reason for persistent uncertainty in this field is methodologic: Reliable techniques for measuring true rates and metabolic sources of DNL in living organisms, including humans, had not been available until recently. It is therefore worth discussing in some detail methods used for measuring DNL.

Indirect calorimetry. Indirect calorimetry, or measurement of gas exchange, has been used for many years as an index of DNL (1,15). When nonprotein respiratory quotient (RQ) is >1.0, net DNL is believed to be occurring in the organism or tissue being studied, since the RQ of DNL is well above 1.0, whereas the RQ of CHO, protein, and FA oxidation are 1.0, ~0.83, and 0.70, respectively (1,16). The problems are that this is a nonmechanistic "black-box" measurement based on many assumptions (16) and, more importantly, that it only can measure net DNL rather than the true unidirectional synthesis of FA. If fat is being both oxidized and synthesized from CHO in the organism concurrently, the net RQ becomes 1.0, and indirect calorimetry reports only that CHO oxidation is occurring. Thus, indirect calorimetry cannot exclude DNL/FA oxidation substrate cycles, which may be important in metabolic regulation (17), and indirect calorimetry cannot quantify true flux rates through DNL. Nevertheless, indirect calorimetric measurements have generally indicated that *net* DNL is unusual in humans, even when fed surplus CHO energy in a single meal or for several days (18–22). Unfortunately, these results could not definitively establish rates of DNL.

Isotopic methods. Dynamic biochemical processes, such as DNL, are most appropriately measured by use of isotopes. Administration of labeled precursors allows newly synthesized molecules to be identified over an experimentally defined time period and permits the relationships among regulatory factors, specific metabolic precursors, and chemical fluxes to be quantified. Measurement of any metabolic process that involves polymerization biosynthesis (combination of repeating monomeric subunits into a polymer) has a fundamental requirement, however (23,24). It is necessary not only to measure how much of the labeled substrate is converted to the polymer of interest, but also to measure the degree of labeling of the true intracellular precursor (the monomeric pool from which the polymer is synthesized). For DNL, the biosynthetic precursor is cytosolic acetyl-CoA (Fig. 1A). How to gain access to the labeling (specific activity or enrichment) of the lipogenic acetyl-CoA pool has presented a long-standing problem for measurement of DNL. The acetyl-CoA pool is particularly complex, in that there are numerous potential metabolic sources of acetyl-CoA whose input varies in a regulated manner according to metabolic conditions. Moreover, cellular acetyl-CoA pools used for different metabolic pathways are compartmentalized and inhomogeneous. For example, acetyl-CoA for hepatic ketogenesis exhibits different labeling than acetyl-CoA for tricarboxylic acid cycle, lipogenesis, and cholesterogenesis in liver (25). It is therefore not possible to infer how much of a labeled substrate has actually entered the lipogenic acetyl-CoA pool relative to unlabeled substrates, or to use surrogate measures of acetyl-CoA (e.g., ketone bodies) to represent the true precursor pool for DNL (26).

The older literature attempting to measure lipid synthesis from ^{14}C-labeled acetate in fact represents an excellent example of the general methodologic principle concerning the importance of measuring precursor pool-specific activity. Several early investigators concluded that insulin plus glucose inhibits cholesterogenesis by hepatocytes, based on lower incorporation of ^{14}C-acetate into cholesterol. It is now clear that these conclusions were entirely wrong because dilution of acetyl-CoA by unlabeled glucose (from activation of glycolysis and pyruvate dehydrogenase activity by insulin) was not accounted for. Cholesterogenesis was in fact stimulated, although total label incorporation was reduced, as would have been apparent if the precursor specific activity had been measured.

An experimental solution to the problem of the lipogenic precursor pool: labeled water incorporation. Awareness of the precursor pool problem 30 years ago led to the development of the $^{3}H_2O$ incorporation technique for measuring DNL and cholesterogenesis (27). This approach solves the problem of the precursor pool by bypassing it. Instead of labeling lipids with a traditional labeled metabolite, a labeled cofactor (H_2O that exchanges with NADPH) is used. Because water mixes freely across almost all biological membranes,

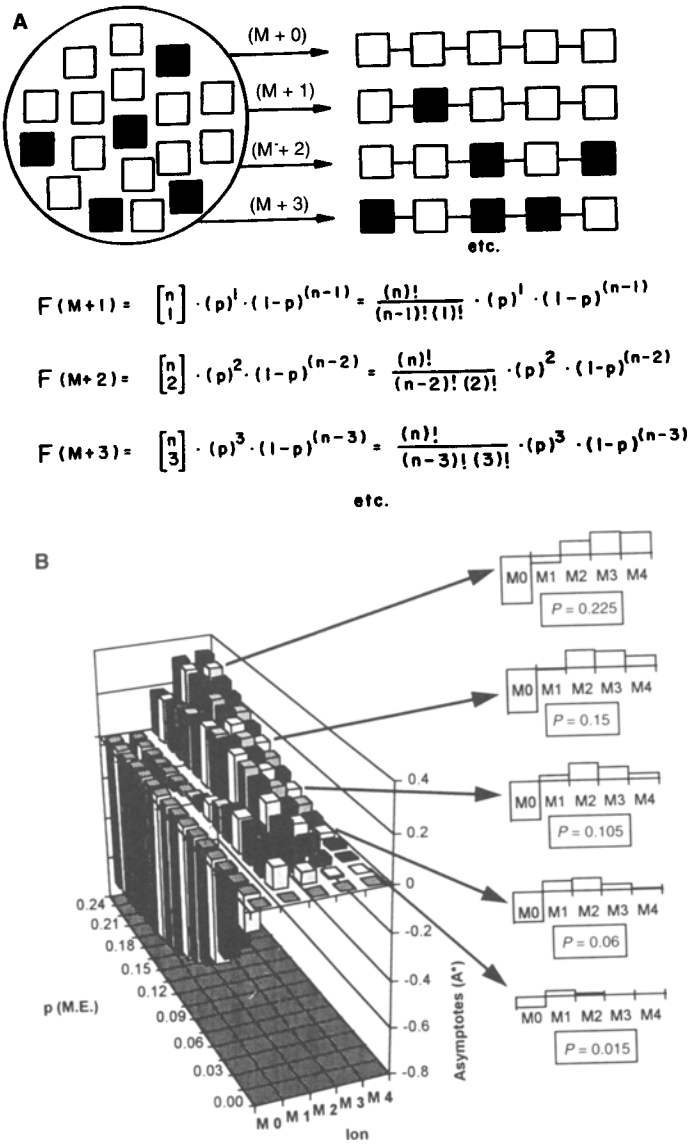


FIG. 1. A) Model of the relationship between isotope enrichment of the monomeric subunits in a precursor pool and the abundance of mass isotopomer species in its polymeric biosynthetic product. Frequency (f) of each isotopomeric species containing *x* labeled subunits [■] out of a total of *n* subunits is calculated from the probability (*p*) that each precursor subunit is labeled, according to the binomial-expansion (shown) (35). B) Three-dimensional histogram showing the effect of varying acetyl-CoA precursor pool enrichment (*p*) on the pattern of mass isotopomer enrichments in a polymer composed repeating acetate submits, in this example, cholesterol. The excess fractional abundances of M_0- to M_4-cholesterol isotopomers from [2-^{13}C]-acetate label are shown. Each cross-sectional slice shown represents the isotopomer pattern characteristic of a particular value of *p*. By analysis of the isotopomer pattern, it is therefore possible to infer the value of *P* (34).

isotopic compartmentalization is considered unlikely, and the specific activity of any body water pool can be used to represent the specific activity of water at the site of synthesis. More recently, ^2H_2O incorporation was pioneered by Jones *et al.* (28–31) for measuring lipid synthesis in humans. Although long considered the gold standard for lipid synthesis, labeled water-incorporation methods are based on a number of assumptions and nonmeasured parameters that may compromise interpretability of results, particularly in human subjects.

Hydrogen is in fact incorporated into lipids both from NADPH and H_2O itself. It is therefore necessary to choose a hydrogen:carbon (H:C) ratio for a newly synthesized lipid molecule that reflects the proportion of H atoms in NADPH that are in equilibrium with cellular water. Unfortunately, the proportion of NADPH hydrogen atoms that derive from water is variable, according to the metabolic sources of NADPH in the cell at a particular time—pentose phosphate pathway, malic enzyme, and so forth. (31). Administration of ethanol, for example, changes the H:C ratio (32). Techniques for estimating the H:C ratio experimentally have also been problematic. Early techniques used the ^{14}C-specific activity of cellular ketone bodies to represent true ^{14}C-acetyl–CoA specific activity (33) and to calculate H:C incorporation ratios. It is now clear that this approach is flawed, since cellular acetyl-CoA pools are highly compartmentalized and differentially labeled (25) and since ketone bodies are synthesized in mitochondria; thus these do not even reflect the cytosolic acetyl-CoA pool in theory. Despite these concerns, a constant standard value for the H:C ratio based on these early *in vitro* studies has typically been used for studies in whole animals and humans (28–30). A recent study using a more rigorous technique for directly measuring the H:C ratio in cholesterol (34), for example found that the ratio was quite variable, ranging from 20 to 30, in contrast to the constant value of 22 typically assumed by researchers. A significant overestimation of cholesterol synthesis would occur if a value of 22 is used for H:C in cholesterol when the true number is 27 or 30. The same considerations apply for DNL as for cholesterol synthesis.

Nevertheless, ^2H_2O incorporation has provided one technique for measuring DNL in humans that makes an attempt to define the true precursor isotope enrichment. Using this approach, Jones *et al.* (28–31) have investigated effects of diet composition and temporal patterns on FA synthesis.

Another experimental solution to the precursor pool problem: mass isotopomer distribution analysis (MIDA). A more recent solution has been developed for measuring polymerization biosynthesis and the true precursor pool, based on the principle of combinatorial probabilities (34–37). Mass isotopomer distribution analysis (MIDA) uses nonradioactive (stable) isotopes with quantitative mass spectrometry. The basic principle of MIDA is that the isotope distribution or labeling pattern of a polymer synthesized from an isotopically perturbed monomeric precursor pool conforms to the binomial (or multinomial) expansion (Fig. 1). The proportion of unlabeled, single-labeled, double-labeled (and so forth) molecular species of a polymer is a function of the probability that each precursor subunit was isotopically labeled (*p*) and the fraction of newly synthesized polymers present (dilution of newly synthesized polymers by preexisting natural abundance molecules). Calculations are somewhat more complex

than analyzing a simple binomial distribution, because carbon in nature contains ^{13}C at an abundance of approximately 1.09% (34); one must therefore calculate the difference between natural and isotopically perturbed distributions (34,36). It can be shown mathematically (34,36), however, that the internal relationship between mass isotopomers (e.g., the ratio between single-labeled and double-labeled molecules) is uniquely determined by p, the true precursor enrichment, independent of dilution of the polymer by unlabeled molecules (Fig. 1B). One can therefore calculate p from the isotope pattern in the polymer. Alternatively, a matrix correction for natural abundance contribution to the labeling pattern (40) or nonlinear best-fit regression equations (41) can be used to calculate p. Once p is known, it is a simple matter to calculate fractional synthesis (f), according to the standard precursor/product relationship (34,36). MIDA and analogous combinatorial probability methods have been used for measuring endogenous synthesis of FA (35–38,42,44), cholesterol (38,45), gluconeogenesis (39), glyconeogenesis (39,46), protein synthesis (47,48) and bile acid synthesis (49) [Kuipers, F., Bandsma, R., Neese, R., and Hellerstein, M.K. (1995) unpublished observation].

MIDA provides a number of advantageous theoretical and practical features for measuring DNL. First, there can be no disputation as to whether the correct acetyl-CoA pool was isolated for the particular lipid product of interest, since calculation is based on the lipid molecule itself. Acetate units in a molecule itself obviously reflect the acetyl-CoA pool from which the molecule was synthesized. Second, metabolic contribution to the lipogenic acetyl-CoA pool from various substrates (glucose, acetate, ethanol, and so forth) can be measured (38,44). Also, precursor pools for synthesis of different lipids can be compared, to determine, for example, whether cholesterol and FA derive from the same precursor pool (38) and thus might compete for substrate, whether *de novo* and chain-elongated FA share a common acetyl-CoA pool (35), or whether the metabolic source of different lipids varies according to physiologic conditions [Kuipers, F., Bandsma, R., Neese, R., and Hellerstein, M.K. (1995) unpublished observation]. Higher-order polymers can also be studied by MIDA; triglycerides can in principle be analyzed as polymers of palmitoyl-CoA, for example, of glycogen as a polymer or glucose subunits. Finally, from the analytic perspective, MIDA requires only a single biochemical isolation and measurement step, performed on the polymer of interest itself. The need for isolating putative biochemical precursors (cellular acetyl-CoA, triosephosphate, and so forth) is obviated. For secreted polymers, MIDA is therefore completely noninvasive (no tissue sample is required); for retained polymers, it is still simpler and less laborious than biochemical isolation of a precursor molecule.

In summary, there now exist two techniques for measuring DNL in humans: MIDA and deuterated water incorporation. This represents significant progress, since only a few years ago no rigorous methods were available. Both techniques use stable isotopes with mass spectrometry, thereby avoiding exposure to radioactivity. MIDA possesses certain advantages over water-incorporation techniques, particularly the ability to characterize metabolic behavior of the intracellular precursor pool (sources, fates, turnover, compartments, and so forth) and freedom from reliance on unmeasured correction factors (the H:C ratio assumed for water-incorporation calculations).

DNL in humans on standard Western diets. Based on these methodologic advances, hepatic DNL has been measured in humans under a variety of experimental conditions. We (35) first measured hepatic DNL in normal men (nonobese, nondiabetic, not overfed, on their standard *ad libitum* diets). The fraction of circulating very low density lipoprotein (VLDL)-palmitate and stearate derived from DNL after a 16-h infusion of [1-^{13}C]-acetate was 1–2% fasted and <5% after 8 h of feeding, whether with a food meal, intravenous glucose (at 7–10 mg/kg/min), or an oral liquid formula (at 7–10 mg CHO/kg/min). Total hepatic DNL was therefore <1 g palmitate per day, a trivial contribution in comparison to typical dietary fat intake (100 g/day or more). Jones and Schoeller (28) have presented a similar estimate of DNL (<2 g/day) using ^2H_2O incorporation. These results support previous conclusions from indirect calorimetry (18–22) and comparison of adipose tissue to diet FA composition (49) that DNL is not an active pathway under usual conditions on a modern (high-fat) diet.

We then measured DNL in young women and obese men (38). Although a significant menstrual cycle variation was observed in women, with three-fold higher values of fractional DNL in the follicular phase compared to the luteal phase, the calculated fat burden imposed by DNL per year was small (a couple of pounds) in comparison to the dietary fat burden (about 100 pounds a year). It is therefore unlikely that DNL explains body-composition differences between men and women. DNL was also slightly elevated in obese, hyperinsulinemic men (38), but again could not account for body-composition differences.

Effects of dietary CHO manipulations. Are there any dietary circumstances where DNL is substantially increased? Schwarz *et al.* (50,51) have measured the effects of oral fructose on DNL. When given at equicaloric doses as glucose (7 mg/kg/lean tissue/min for 6 h), fructose caused an almost twenty-fold increase in fractional DNL above fasting values, whereas glucose had almost no effect. Close to 30% of circulating triacylglycerol (TG)-palmitate came from DNL after this rather moderate dose of fructose. Even so, only a small proportion of the fructose load was converted to fat (less than 5%). Hudgins *et al.* (52,53) (Rockefeller University) have studied DNL in normal subjects placed on very-low-fat/high-CHO eucaloric liquid formula diets (10% fat, 70% CHO) for 25 days under metabolic ward conditions. Fractional DNL measured by MIDA increased to about 20% (fasted) and 30% (fed). A nonisotopic approach (dilution of VLDL-TG 18:2 relative to dietary and adipose FA 18:2, which were matched in the study) confirmed a substantial contribution from DNL to FA in VLDL-TG. Absolute hepatic DNL calculated by MIDA was nevertheless rather modest (e.g., less than 5 or 10 g fat synthesized per day) on these eucaloric, very low fat diets.

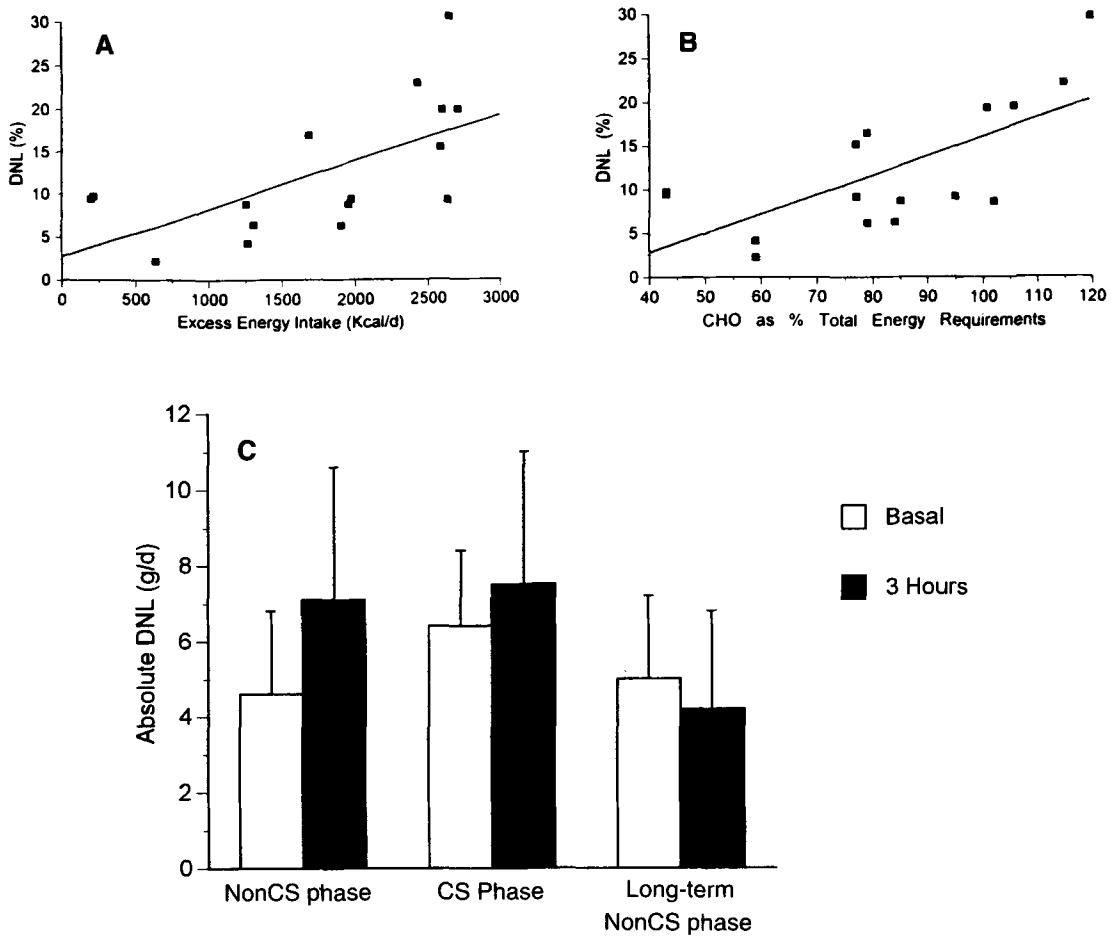


FIG. 2. Effect of *ad libitum* overfeeding on *de novo* lipogenesis (DNL) in humans, and interaction with cessation of cigarette smoking (CS): A) relationship between excess energy intake (kcal/day above estimated daily requirements) and fractional DNL ($r^2 = 0.39$, $P < 0.01$); B) relationship between percent of daily energy requirements provided by carbohydrates (CHO) and fractional DNL ($r^2 = 0.47$, $P < 0.01$); C) absolute DNL during CS and non-CS phases. Basal refers to fasting value. 3 hours refers to value after 3 h of CS of nonsmoking.

Effects of overfeeding. It can legitimately be argued that these conditions do not represent a true test of DNL if the pathway functions as a disposal route for *surplus* CHO energy. It might be necessary to overfeed CHO—that is, provide surplus energy in the form of CHO to the whole body—to induce DNL. It should be noted that previous indirect calorimetric data have generally failed to show much net DNL in overfed humans, at least on a mixed diet, unless ingested at enormous excess (20–22). Our first data in this area came out of a study in which we were asking whether the weight gain after cessation of cigarette smoking (CS) could be due in part to increased DNL. To address this question, Hellerstein *et al.* (43,55) studied smokers under metabolic ward conditions during a week of CS, then a week of no CS, and repeated measurement following four more weeks of abstention from CS. We evaluated two dietary conditions—one in which a constant metabolic diet was given (43) and one in which *ad libitum* intake was allowed (54). DNL was not increased by cessation of CS per se. Several subjects on *ad libitum* diets overate to a remarkable extent, however (54). Some subjects consumed 5,500–6,000 kcal/day, with 3,000–4,000 kcal from CHO. Fractional DNL was substantially increased in the *ad libitum* diet group, and a strong positive correlation was observed between calculated surplus energy intake or CHO energy intake and fractional DNL (Figs. 2A and 2B), implying that a dose–response relationship exists. When absolute DNL was calculated, however, <10 g/day of FA (palmitate and stearate) was synthesized even in subjects with the highest DNL rates (Fig. 2C). Synthesis of new fat was therefore quantitatively minor compared to ingestion of dietary fat (>100 g/day) in these individuals (54).

We have since followed up on these observations by administering defined diets containing up to 50% excess or deficit of energy as CHO or fat for 5-day periods (55). Fractional DNL was found to be a remarkably sensitive index of recent CHO energy intake—increasing more than ten-fold on

surplus CHO and becoming unmeasurably low on CHO-deficient diets, while exhibiting a linear dose–response relationship to CHO intake. DNL did not increase above baseline values on 50% excess *fat* calories. CHO intake over the preceding 5 days could in fact be diagnosed with near 100% sensitivity and specificity by DNL, which therefore represented an objective biomarker of recent CHO energy intake. Nevertheless, absolute hepatic DNL accounted for <5 g of fat synthesized, even on 50% surplus-CHO diets (55).

Several obvious questions are raised by these findings. First, where do excess CHO calories go, if not into DNL? Second, are surplus CHO calories therefore "free"—i.e., do they not contribute to body-fat stores? And third, what may be the function of DNL, if storage of surplus CHO energy as fat is not a quantitatively important process? The answers to the first two questions are clear from our substrate oxidation measurements (43,55) and those of previous investigators (20–22). Addition of CHO in excess to a mixed diet results in preferential oxidation of dietary CHO and sparing of dietary fat from being oxidized. Thus, surplus CHO may not itself be converted to fat, but it nevertheless will result in increased body-fat stores by preventing dietary fat from being oxidized. The answer to the second question is therefore no, CHO calories are not free. Nonprotein RQ rises to close to 1.0 on surplus CHO intake even after an overnight fast (22,55), indicating nearly complete (>90%) suppression of fat oxidation.

The distinction between excess CHO being converted to fat vs. sparing oxidation of fat is not just a technicality, however, despite the shared consequence of either process on body-fat stores. Attempts to understand or prevent adverse effects of overfeeding depend on establishing the precise metabolic fate and consequences of surplus energy intake. For example, some health-food entrepreneurs have proposed use of an inhibitor of DNL, (-)hydroxycitrate, for treatment of obesity. If this is intended to reduce storage of fat by reducing its synthesis from CHO, our results make it clear that the strategy will not be quantitatively effective. Also, we observed that short-term changes in CHO intake and energy balance exerted striking effects on fasting hepatic glucose production (HGP) in normal humans (55) and patients with non-insulin-dependent diabetes mellitus (NIDDM) (56). Increased HGP accounted for most of the increased CHO oxidation that occurred in the fasted state in subjects who had been overfed CHO for 5 days. Increased fasting HGP therefore serves a metabolic function in the context of a system where surplus CHO energy ingested during the day is not immediately converted to fat for later oxidation at night (i.e., a cyclic system with RQ >1.0 and <1.0 during the day and night, respectively), but instead is stored as liver glycogen, which provides fuel for oxidation at night (a noncyclic system with a steady RQ close to 1.0 during both day and night). Since increased HGP is a major contributing factor to insulin resistance and NIDDM (57), the inability of humans to use the DNL pathway as a disposal route for excess CHO in mixed diets may be an important factor in the etiology of NIDDM.

Studies of overfeeding on mixed diets cannot establish what would happen to DNL if a true surplus of CHO energy were present in the absence of dietary fat—i.e., if a very-low-fat surplus-CHO diet were administered for long enough to saturate the maximal whole body glycogen storage capacity. This experiment has yet to be done under controlled conditions, as will be explained later.

What are the functions of DNL? If DNL does not generally act as a sink for disposal of overflow CHO energy, what then are its functions? There are several possible answers to this question.

(1) DNL has no physiologic functions, but is a vestigial pathway in humans. This seems unlikely a priori, in view of the complex machinery for DNL and the regulation that clearly occurs *in vivo* as well as *in vitro*.

(2) DNL is important *in utero* for embryonic development, but becomes unimportant postnatally when exogenous FA can be ingested and absorbed (i.e., it is a vestigial pathway developmentally). This is an interesting possibility that has some support. The apparently limited ability of FA to cross the placenta combined with enormous lipid demands of the developing fetus (especially for myelination in the central nervous system and deposition of subcutaneous fat during the third trimester of pregnancy) suggest that DNL may be very active *in utero*. One model is that after delivery, ingestion of a high-fat diet (in breast milk) suppresses DNL. This has not been established in humans, however.

Two recent observations provide some indirect evidence on this hypothesis. The first is from a mouse knock-out model (58). Knockout of the gene for apolipoprotein B results in lethality *in utero*. It may be speculated that inability to transport lipid synthesized in the fetal liver to the central nervous system is responsible. The second observation is clinical (Chwals, W., personal communication). Premature infants who are stressed can exhibit remarkably high RQ values (1.10–1.20) when treated with intravenous nutrition, consistent with massive *net* DNL. Perhaps this reflects persistence of the prenatal pattern, in the face of relative energy excess. Both these speculations must be tested experimentally, however.

(3) DNL is important on very-low-fat diets but is unimportant on the unnaturally high-fat modern diet (i.e., is a vestigial pathway culturally). As noted above, DNL is at least qualitatively increased on eucaloric very-low-fat diets in normal adults (52,53). It will be of interest to establish whether large short-term adaptations in DNL do occur in response to day-to-day variations in energy balance in subjects on a very-low-fat diet. If so, DNL may function in the energy balance economy but only if modulation of dietary fat oxidation is not available as a first metabolic option.

(4) DNL is in fact important for storage of surplus CHO energy, but the proper experiment has yet to be carried out. Effects of true CHO energy surplus (CHO in excess of total energy expenditure, for long enough to saturate glycogen stores and in the presence of a low-fat diet) on DNL have not

yet been determined. The simple expedient of replacing fat with CHO in the fuel mixture or expanding whole body glycogen stores would no longer be an option for disposal of surplus CHO energy in this setting. Another possibility to consider is that until now the wrong tissue has been evaluated. Perhaps adipose tissue can become a significant site of DNL under certain circumstances, in which case adipose biopsies for measurement of DNL would be required. Some indirect evidence has been presented that, at least during intravenous feeding of sick patients, adipose DNL may become active [Wolfe, R.R. (1994) personal communication].

(5) DNL serves regulatory roles, perhaps as a signal of CHO availability in tissues. Malonyl-CoA concentrations and perhaps DNL influence fuel selection (FA oxidation vs. reesterification) in liver and muscle (2–4) and secretion of insulin in the pancreatic ß-cell (59). The sensitive qualitative response of DNL to CHO energy balance (55) may therefore influence fat balances not through the input of new FA but by controlling FA oxidation.

(6) DNL is necessary for synthesis of special pools of lipids. The metabolic source of the myristate used for acylation of proteins (60), for example, is not known. Is there a dietary myristate requirement, or is this FA synthesized *de novo*? This will be interesting to establish and is amenable to experimental study using the techniques previously described.

(7) DNL becomes important in specialized tissues or under special conditions. The lactating mammary gland appears to have a very active DNL pathway, at least in animal models (14). The metabolic source of lipids in breast milk of humans has not yet been established, however. Brown fat also may have extremely active DNL, which may be one mechanism of adaptive thermogenesis in this organ (Danforth, E., personal communication). Ob/Ob mice exhibit up to fifty-fold increased DNL (61), although contribution to their increased body-fat stores is not clear.

Does DNL have a role in disease? There are several disease conditions to which DNL might contribute. Some speculations are as follows: (i) hypertriglyceridemia (especially CHO-induced) (64–66), (ii) wasting syndromes [e.g., acquired immune deficiency syndrome (AIDS)] and partitioning of nutrients (42,69–73), (iii) alcoholic liver disease (fatty liver) (68), (iv) membrane FA composition (e.g., insulin resistance) (75). Hypertriglyceridemia, especially in response to high-CHO diets (62–64) may be influenced by DNL either directly (through addition of FA to VLDL-TG) or indirectly (through inhibition of tissue FA oxidation and stimulation of hepatic reesterification). Alcoholic liver disease is preceded by fatty infiltration of the liver, which could be at least in part, a consequence of ethanol-stimulated DNL or secondary inhibition of tissue FA oxidation (65,66). Body-composition abnormalities characteristic of patients with wasting in AIDS— preservation of body fat relative to lean body mass (67) and repletion of body fat rather than lean body mass during refeeding (68–70)—may be influenced by DNL. We reported (42) that DNL is paradoxically elevated in patients with weight loss due to AIDS. Moreover, the presence of increased DNL predicted poor lean-tissue response to nutrient supplementation (71), consistent with either a causal role for DNL in altered nutrient partitioning or a role for DNL as a marker of underlying cytokine abnormalities (72). Finally, altered membrane FA composition has been associated with a number of clinical conditions, including insulin resistance (73). If DNL alters qualitative FA composition in susceptible individuals, it could contribute to disease without being an important pathway quantitatively in the whole body.

In conclusion, before a process can be discussed, it must be amenable to measurement. This is now possible for DNL by two methods: MIDA and deuterated water incorporation. Based on these direct isotopic measurement techniques, a somewhat surprising model has emerged of how the human body handles excess CHO energy. DNL does not seem to be a highly traveled pathway, at least in normal adults on typical Western diets. A more detailed understanding of the quantitative contribution from DNL and testing of other potential functions of DNL are required. These remaining areas of uncertainty should now be accessible to experimental analysis, however, based on the recent methodologic advances.

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In Vivo ^{13}C Nuclear Magnetic Resonance: Applications and Current Limitations for Noninvasive Assessment of Fatty Acid Status

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ABSTRACT: As a noninvasive method, *in vivo* ^{13}C nuclear magnetic resonance has potentially important applications in understanding the metabolism of long chain fatty acids in organs of living humans. At present, this methodology is most advanced for research on glucose utilization. However, the main ^{13}C signals visible *in vivo* are from fatty acids in adipose tissue and the olefinic signals can be used to noninvasively estimate adipose tissue content and relative dietary intake of polyunsaturates and monounsaturates. The low natural abundance of ^{13}C improves the utility of this isotope for fatty acid tracer studies. Due to excessive signal broadening, uniform ^{13}C-labelling seems to have limited application in *in vivo* fatty acid studies. Tracer fatty acids with ^{13}C enrichment at a specific carbon position, i.e., [3-^{13}C]γ-linolenate, appear to be the most useful for *in vivo* tracer studies. Development of methods permitting resolution of ^{13}C enrichment in structural lipids of lean tissues will be an important breakthrough which may make human tracer studies feasible and worthwhile.
Lipids 31, S-127–S-130 (1996).

Our aim here is to review the background to *in vivo* ^{13}C nuclear magnetic resonance (NMR) spectroscopy and to describe current efforts to apply this methodology to noninvasive measurement of fatty acid status and metabolism. The purpose of this approach to determining fatty acid metabolism is to address the need for noninvasive methodology applicable to studying organ composition and metabolism, especially in infants. The technical challenges to obtaining meaningful data are significant but the recent availability of isotopically-labelled tracer fatty acids developed specifically for *in vivo* NMR studies has helped make these studies more feasible.

Using naturally abundant ^{13}C, *in vivo* ^{13}C NMR experiments have been done in experimental animals and humans for 15 yr (1–4). This methodology has recently shown very useful application in noninvasively monitoring the metabolism of glucose in both human brain (5) and skeletal muscle (6). Relatively large doses of ^{13}C-glucose are required for these human studies and, as in other clinical investigations, blood samples are taken for routine assays, i.e., glucose and insulin measurements in this case. Otherwise, these experiments are noninvasive and provide information on the time course and route of metabolism of glucose in individual organs. Aside from the importance of the metabolic questions they address, these experiments demonstrate a clear application of *in vivo* ^{13}C NMR in noninvasive studies of glucose metabolism in humans of all ages. The goal in *in vivo* NMR studies of fatty acid metabolism is to have a similar degree of success as has been achieved in the work on glucose metabolism but the impediments are different and perhaps more daunting.

Principles of in vivo *^{13}C NMR. In vivo* ^{13}C NMR methods are well-established in animal and human models and have been in use for at least a decade to monitor glucose metabolism (1–7). For research in humans, a horizontal bore magnet is required and most of these magnets have a clear internal bore of about 1 m. The subject lies prone and the whole body is inserted into the magnet on a sliding bed. Smaller magnets with an internal bore of 15–40 cm have also been used to acquire spectra from the limbs of human subjects (8). For animal models, vertical bore magnets with a field strength of up to 9–10 Tesla are available and have the advantage of much better signal-to-noise than can be achieved at lower field strengths.

Aside from magnetic field strength and surface coil design, three important considerations affect the quality of *in vivo* NMR data: the sensitivity of NMR to detect a particular nucleus and the abundance and mobility of the molecule in question. NMR sensitivity is an inherent feature of the nucleus itself and, for the main nuclei of biological interest, sensitivity is significantly higher for protons and ^{31}P than for ^{13}C. Protons and ^{31}P are both essentially 100% abundant but the NMR-sensitive isotope of carbon (^{13}C) is only about 1.1% abundant. Hence, detection of ^{13}C is considerably more difficult than detection of protons or ^{31}P. The "mobility" of a

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Abbreviations: NMR, nuclear magnetic resonance; PUFA, polyunsaturated fatty acids.

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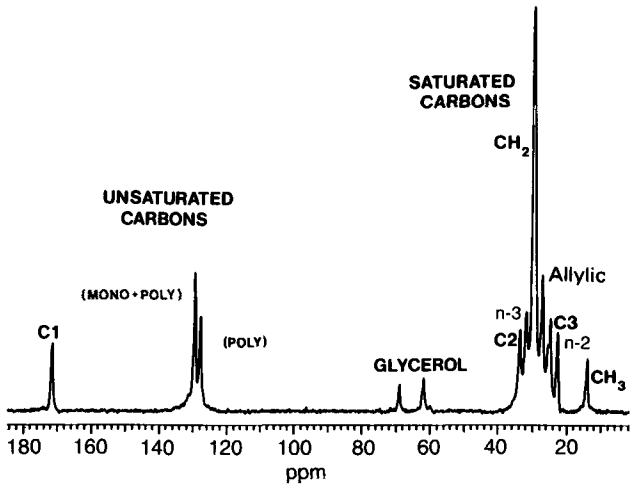


FIG. 1. *In vivo* ^{13}C nuclear magnetic resonance spectrum obtained at 4.7 T. The spectrum is of epididymal adipose tissue in a living rat. The main signal clusters are identified. Reprinted from Reference 9, with permission.

molecule, i.e., its ability to undergo tumbling motion, also determines how easy it is to detect and resolve different molecules by NMR. Molecules in biological matrices are less mobile than those in solution so, even under optimal conditions, *in vivo* NMR always has considerably lower resolution of the available information than can be achieved with samples in solution. Small molecules are more mobile than large molecules so free amino acids are easier to detect *in vivo* than proteins. Fatty acids are mainly deposited in tissues as phospholipids or as triglycerides. Triglycerides are mostly present as liquid droplets and exhibit more mobility than phospholipids which are mostly constrained by the structure of biological membranes. Hence, fatty acids in the free form (unesterified) or esterified into triglycerides are easier to detect *in vivo* than those present in phospholipids.

In vivo *^{13}C NMR of fatty acids.* The main ^{13}C NMR signals visible in *in vivo* spectra are from fatty acids in subcutaneous fat (Fig. 1). ^{13}C NMR signals from fatty acids cluster in five main regions: (i) the carboxyls around 175 ppm, (ii) the olefinic carbons around 130 ppm, (iii) signals for glycerol at 62 and 69 ppm, (iv) the aliphatic carbons between 20 and 35 ppm and (v) the terminal methyl carbons at 14 ppm (Fig. 1). In adipose tissue, there is little detectable influence of different dietary fatty acids on carboxyl, glycerol, aliphatic, or terminal methyl carbon resonances, but the olefinic region is sensitive to differences in fatty acid unsaturation (9,10). *In vivo*, the olefinic region consists of a pair of peaks centered at about 130 to 128 ppm. These two peaks overlap considerably, but it is generally possible to detect the break between them which is necessary to derive information about the degree of fatty acid unsaturation in the adipose tissue.

In theory, all unsaturated fatty acids contribute to these two peaks but in practice, adipose tissue is rich in oleate (18:1n-9) and linoleate and the total of the other unsaturated fatty acids rarely accounts for more than 5–8% of adipose tissue fatty acids (11). The "outer" olefinic carbons of linoleate (C_9,C_{13}) give rise to most of the signal at 128 ppm while the "inner" olefinic carbons of linoleate (C_{10},C_{12}) plus the two unsaturated carbons of oleate (C_9,C_{10}) both contribute to the signal at 130 ppm. On the assumption that these two peaks are due only to the four unsaturated carbons in linoleate and the two unsaturated carbons in monounsaturates represented by oleate, linoleate would contribute equally to both peaks and, therefore, subtraction of the integrated peak area at 128 ppm from that at 130 ppm gives the relative area for monounsaturates by difference. Hence, an estimate of the ratio of polyunsaturated fatty acids (PUFA) to monounsaturates in adipose tissue can be determined noninvasively (9).

In practice, the only significant exception to the assumption that the olefinic region of *in vivo* spectra of adipose tissue arises from linoleate and monounsaturates comes from the two main PUFA in fish—eicosapentaenoate (20:5n-3) and docosahexaenoate (22:6n-3). These two fatty acids are highly unsaturated and, if present in adipose tissue at > 2–3%, contribute significantly to the "inner" olefinic signals at 128 ppm (9). However, these two fatty acids are not normally consumed in high enough amounts to accumulate significantly in human adipose tissue (11) so, under usual dietary conditions, they would generally have a minor influence on the ratio of PUFA to monounsaturates.

The responsiveness of the olefinic region in *in vivo* ^{13}C NMR spectra to dietary fat (9) and the knowledge that human adipose tissue composition is directly related to fatty acid intake (12) and not to fatty acid synthesis (13) has stimulated interest in determining PUFA intake based on the relative peak areas at 128 and 130 ppm. Cystic fibrosis (8) and vegetarianism (14) have both been shown to significantly alter the PUFA/monounsaturate peak ratio in ^{13}C NMR spectra of subcutaneous adipose tissue obtained noninvasively. In each case, comparisons were made with the fatty acid composition of adipose tissue determined by gas chromatography so the level of confidence in the *in vivo* NMR is good. These are important steps towards applying ^{13}C NMR in subjects from which it is becoming ethically less acceptable to sample blood or adipose tissue, i.e., babies.

Fatty acids in lean tissues are mostly in relatively immobile phospholipids and, therefore, organs such as liver generate much less overall signal than the adipose tissue which comes between the liver and the surface coil. Isolation of samples of lean tissues clearly shows that most abdominal tissues or organs normally have little or no NMR-visible fatty acids signals (9). In principle, this could work out to be an advantage for studies of fatty acid metabolism because if the intensity of the adipose tissue signal could be reduced or eliminated, a low natural abundance ^{13}C NMR signal in the underlying organ of interest would allow one to more easily detect the presence of a tracer fatty acid.

At present, it is not possible to resolve more information from *in vivo* ^{13}C NMR spectra obtained in human subjects than total PUFA and their ratio to monounsaturates and saturates in subcutaneous adipose tissue. Even though there appears to be a good correlation between data derived from

NMR spectra and measurements of fatty acids in needle biopsies of adipose tissue by gas ghromatography, the *in vivo* NMR approach provides relatively crude information. Very few centers have access to NMR spectrometer equipped for *in vivo* ^{13}C studies in humans and, in the infant population in which this method theoretically and ethically has the most application, reduction of data quality due to proton coupling and movement of the patient reduces its usefulness. If it is ethically acceptable to obtain a blood sample for diagnostic purposes in appropriate cases, it should also be acceptable to obtain a needle biopsy of 5–20 mg of fat which can then be analysed by gas chromatography to yield far more useful and detailed information than a crude ratio of the three main groups of fatty acids (11).

Tracing fatty acid metabolism. Using ^{13}C as a tracer isotope takes advantage of the wide signal dispersion in carbon spectra and the numerous different bond environments (carboxyl, olefinic, aliphatic, allylic, doubly allylic) in different fatty acids and lipids thereby potentially providing extensive detail about the "molecular" location of ^{13}C enrichment. This has been clearly demonstrated in *in vitro* NMR studies of ^{13}C-PUFA metabolism (1,2,15) but *in vivo* studies are less advanced. In our experience, the quality of ^{13}C enrichment data obtained *in vivo* is dependent on the type of ^{13}C tracer used.

If the tracer is uniformly (U) labelled, ^{13}C mass/dose could be as much as 18–20 times that of a tracer labelled with ^{13}C at a specific carbon. This may enhance overall detection or permit lower doses to be used. Unlike labelling at a single carbon (typically C-1), this also means that the metabolism of the entire molecule can be traced rather than assuming one tracer carbon, i.e., C-1, represents the metabolism of the entire molecule. ^{13}C-^{13}C coupling is not a significant problem if only one carbon (or two adjacent carbon sites) is enriched, but with U-^{13}C labelling this is a problem *in vivo* because the resulting multiplets overlap sufficiently so that the molecular location of the ^{13}C is harder to determine accurately. This is especially true in lean tissues such as brain in which the fatty acid signals are poorly resolved to start with due to their motion being constrained within membranes. Furthermore, in the case of using an orally-dosed ^{13}C PUFA precursor in suckling rats, the ^{13}C is widely dispersed in brain lipid components such as arachidonate, cholesterol, palmitate (16:0) and oleate (18:1n-9) (2). This generalized incorporation of ^{13}C into brain lipids is readily detected *in vivo* by the improved signal/noise from the presence of the additional ^{13}C in brain lipids (2). However, the ^{13}C labelling of specific carbons of brain cholesterol is not visible *in vivo* even though it is well-resolved by *in vitro* NMR techniques (15) and is quantitatively significant (2). Thus, in our limited experience, it is not yet possible to use *in vivo* NMR to determine the specific lipid or fatty acid location of a U-^{13}C fatty acid tracer in a lean tissue without subsequent lipid extraction and further analysis *in vitro*.

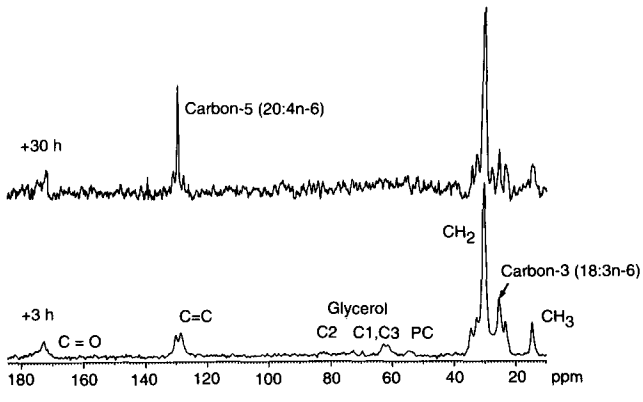


FIG. 2. *In vivo* ^{13}C nuclear magnetic resonance spectra of the abdominal region of living rat pups (10 d old) acquired at 7.4 T in a vertical bore magnet. Control spectra are comparable to those shown in Figure 1. The C-3 region (24.7 ppm) is seen to increase in these spectra obtained 3–30 h after dosing with [3-^{13}C]γ-linolenate. At 3 h post-dosing, no change is visible in the olefinic region where C-5 of arachidonate is located. By 30 h, a significant amount of the γ-linolenate has been metabolized to arachidonate and the ^{13}C-enriched carbon (originally C-3) has become an olefinic carbon and causes the resulting increase in the signal at 128.9 ppm.

Locating the ^{13}C at a specific carbon in the fatty acid tracer greatly improves the chance of seeing the actual molecular site of labelling *in vivo*. If the ^{13}C-enriched carbon is at a CH_2 position which is then desaturated to an olefinic carbon during the fatty acid's metabolism, the extent of that metabolism is, in principle, detectable *in vivo*. For example, using *in vivo* ^{13}C NMR, [3-^{13}C]γ-linolenate (18:3n-6) is visible in the stomach and liver of the suckling rat (Fig. 2). Since the ^{13}C moves from C-3 to C-5 (an olefinic carbon) when the γ-linolenate gets metabolized to arachidonate, the resulting shift in resonance is considerable and is visible *in vivo*. Thus, it is possible to use ^{13}C-labelled fatty acids which are labelled at appropriate and specific carbons to noninvasively trace metabolism of the precursor PUFA and its conversion to the main longer chain metabolite, i.e., arachidonate. It is still not yet clear whether only fatty acids present in freely mobile lipids, i.e., free fatty acids or triglycerides, are visible *in vivo*, but these types of experiments should facilitate answering this important question.

Conclusion. The goal of detecting tracer fatty acids or their products in organ lipids of living human subjects by *in vivo* ^{13}C NMR is worthwhile because it represents one of the few approaches by which we can learn how dietary PUFA are utilized in humans and therefore how best to develop therapeutic strategies for their effective application in chronic human disease. Theoretically, this methodology would be particularly useful for understanding the utilization of dietary PUFA in infants because the development of the brain occurs largely in the first two years of life and many infants are still exclusively fed formula milks the composition of which may not be ideal for optimal cognitive and behavioural development. However, at present, the objective of tracing fatty acid metabolism in living human subjects remains elusive. Tracer fatty acids with high ^{13}C-enrichment at a carbon which undergoes desaturation have provided the first clear indication that this methodology is feasible (Fig. 2). This has only been achieved

at high magnetic field strength and in suckling rat pups receiving a relatively large dose of tracer. If new methods can be developed which reduce the background signal from adipose tissue or facilitate extraction of signals in more rigid lipids (phospholipids, cholesterol), the goal of experimentally monitoring fatty acid metabolism in living humans may hopefully be attained.

ACKNOWLEDGMENTS

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Effect of Dietary α-Linolenic Acid Intake on Incorporation of Docosahexaenoic and Arachidonic Acids into Plasma Phospholipids of Term Infants

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ABSTRACT: The fractional conversion rates of plasma phospholipid α-linolenic acid (18:3n-3) and linoleic acid (18:2n-6) to docosahexaenoic acid (22:6n-3) and arachidonic acid (20:4n-6), respectively, and the fractional rates of incorporation of 22:6n-3 and 20:4n-6 into plasma phospholipids were determined in 27 healthy 3-wk-old term infants who had received formulas with ~16% of fat as 18:2n-6 and 0.4% (n = 6), 1.0% (n = 11), or 3.2% (n = 10) as 18:3n-3 from birth. The infants were given a single dose of both [U-^{13}C]18:2n-6 and [U-^{13}C]18:3n-3 with a feeding, and blood samples were collected 8, 12, and 24 h afterward for determination of the isotopic enrichments of the [M + 18] isotopomers of plasma phospholipid fatty acids by negative chemical ionization gas chromatography/mass spectrometry. A simple precursor/product compartmental model was used to estimate fractional rates of conversion and incorporation. All infants converted 18:3n-3 to 22:6n-3 and 18:2n-6 to 20:4n-6. Although the fractional rate of conversion of 18:3n-3 to 22:6n-3 did not differ among groups, the fractional rate of incorporation of 22:6n-3 into the plasma phospholipid fraction was greater in infants who received 3.2% vs. 0.4% or 1.0% 18:3n-3 (4.1 ± 2.2 vs. 1.6 ± 1.5 or 2.0 ± 1.0% of the plasma phospholipid 22:6n-3 pool daily). The fractional rate of conversion of 18:2n-6 to 20:4n-6 was less in infants who received the 3.2% 18:3n-3 intake (0.4 ± 0.3% of the plasma phospholipid 18:2n-6 pool daily vs. 1.1 ± 0.7% and 0.8 ± 0.5% in those who received 0.4 and 1.0% 18:3n-3, respectively). The fractional rate of incorporation of 20:4n-6 into plasma phospholipid also was less in the 3.2% vs. the 0.4 and 1.0% 18:3n-3 groups (2.7 ± 1.4% vs. 5.9 ± 2.6 and 4.4 ± 1.7%, respectively, of the plasma phospholipid 20:4n-6 pool daily).
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The consistent finding of higher docosahexaenoic acid (22:6n-3) and arachidonic acid (20:4n-6) levels in plasma and tissue lipid fractions of infants fed either human milk, which contains these fatty acids, or formulas supplemented with these fatty acids vs. formulas containing only their precursors is frequently interpreted as evidence that infants either cannot convert the precursor essential fatty acids [α-linolenic (18:3n-3) and linoleic acid (18:2n-6)], respectively, to 22:6n-3 and 20:4n-6 or have limited capacities to do so. Recent studies have shown that both term and preterm infants can convert precursors to products (1,2), but whether conversion is adequate to meet the considerable needs of 22:6n-3 and 20:4n-6 by developing tissues remains unknown. Further, while a high intake of either 18:3n-3 or 18:2n-6 relative to the other is known to inhibit desaturation and elongation of the other (3,4), little quantitative information about this issue is available. Recently, we studied effects of 18:3n-3 intake, or the intake ratio of 18:2n-6/18:3n-3, on plasma and erythrocyte fatty acid patterns, growth and visual development of term (5) and preterm infants (6). In a subset of these infants, we also attempted to quantitate fractional rates of conversion of 18:3n-3 to 22:6n-3 and 18:2n-6 to 20:4n-6 as well as fractional rates of incorporation of 22:6n-3 and 20:4n-6 into the plasma phospholipid fraction. Calculated fractional rates of conversion and incorporation of this subset of 3-wk-old term infants who had received 18:2n-6 intakes of 16% of total fat and 18:3n-3 intakes of 0.4, 1, or 3.2% of total fat from birth are reported here.

MATERIALS AND METHODS

Subjects. Twenty-seven term neonates participating in the previously mentioned study (5) and assigned at birth to receive a formula with ~16% of fat as 18:2n-6 and 0.4% (group A, n = 6), 1.0% (group B, n = 11), or 3.2% as 18:3n-3 (group C, n = 10) were selected randomly at the time of initial enrollment to participate in the studies reported here. The three groups were comparable with respect to gestational age, birth weight, head circumference, gender, and ethnic background, as well as age and weight at the time of study. Except for 18:3n-3 content, nutrient contents of the formulas were identical and similar to those of Enfamil® (Mead-Johnson Nutritionals, Evansville, IN), i.e., 9% of energy as protein, 41% as lactose, and 50% as fat. The study was approved by the Institutional Review Boards of Baylor College of Medicine (Houston, TX) and Harris County Hospital District (Houston, TX). Written, informed parental consent was obtained for all infants enrolled.

At 3 wk of age, each infant was given from 25–40 mg/kg of [U-^{13}C]18:2n-6 and 20–25 mg/kg of [U-^{13}C]18:3n-3

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methyl esters (95–99% chemical purity, 99% isotopic purity; Martek Biosciences Corp., Columbia, MD). Labeled fatty acids were added to a small aliquot of formula, the mixture was sonicated for 60 min, and the sonicated mixture with labeled fatty acids intact (based on periodic assessments) was fed to the infant immediately by nipple. To minimize loss of labeled fatty acids, additional aliquots of formula were added to the sonication vessel before being fed to the infant.

Blood samples were collected in EDTA tubes 8, 12, and 24 h after administration of the labeled fatty acids; plasma and erythrocytes were separated immediately and stored at −70°C until processed further. Plasma phospholipids were separated as described by Hamilton and Comai (7), and fatty acids of this fraction were derivatized to pentafluorobenzyl esters (8) using 1 mL of 0.1M tetrabutylammonium phosphate as buffer and 400 μL of 0.13 M α-bromo-2,3,4,5,6-pentafluorotoluene in methylene chloride as reagent (80°C for 1 h).

Enrichment of the [M + 18] isotopomers of the pentaflurobenzyl esters was determined by negative chemical ionization gas chromatography/mass spectrometry (Hewlett-Packard 5989 mass spectrometer equipped with a Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA), using selected ion monitoring. Samples were eluted on capillary columns, and chromatographic peaks were identified both by comparison of retention times to those of fatty acid standards (Sigma Chemical Co., St. Louis, MO) and by mass determination. Isotopic enrichments (precision, 1–5%) were expressed as tracer/tracee ratios.

A simple precursor/product compartmental model was used to quantitate fractional rates of conversion of plasma phospholipid 18:3n-3 and 18:2n-6, respectively, to 22:6n-3 and 20:4n-6 and fractional rates of incorporation of 22:6n-3 and 20:4n-6 into the plasma phospholipid fraction. Parameters of the compartmental model were computed with the Simulation Analysis and Modeling program (SAAM; University of Washington, Seattle, WA).

In this model (Fig. 1), compartments M_1 and M_2 represent relative concentrations of precursor and product fatty acids in the plasma phospholipid fraction as determined by conventional gas chromatography (9). The decline in precursor enrichment (Fig. 2) secondary to some combination of exchange, oxidation, and/or storage is expressed by the rate constant $k_{0,1}$; that secondary to conversion of the precursor to the longer-chain, more unsaturated product(s) is expressed by the rate constant $k_{2,1}$. Thus, the fractional rate of conversion of the precursor can be calculated as: $k_{2,1}/(k_{0,1} + k_{2,1}) \times 100$. Since the phospholipid content of neither the precursor nor product changes appreciably over the period of study, it is assumed that the fractional rate of incorporation of the product into the plasma phospholipid fraction is equal to its fractional catabolic rate, expressed by the rate constant $k_{0,2}$. The fractional rate of incorporation, of course, is related to the fractional rate of synthesis of the product. All rate constants can be derived from the exponential curves of the decline in precursor enrichment and the increase in product enrichment observed in each infant over the 24-h period of study (Fig. 2). Because behavior of tracer is assumed to be the same as for tracee, the rate constants calculated from changes in enrichment of the labeled precursors and products are assumed to be the same for the total precursor and product pools.

Statistically significant differences among groups ($P < 0.05$) in fractional rates of conversion of precursor to product and incorporation of product into the plasma phospholipid fraction were detected by analysis of variance followed, if indi-

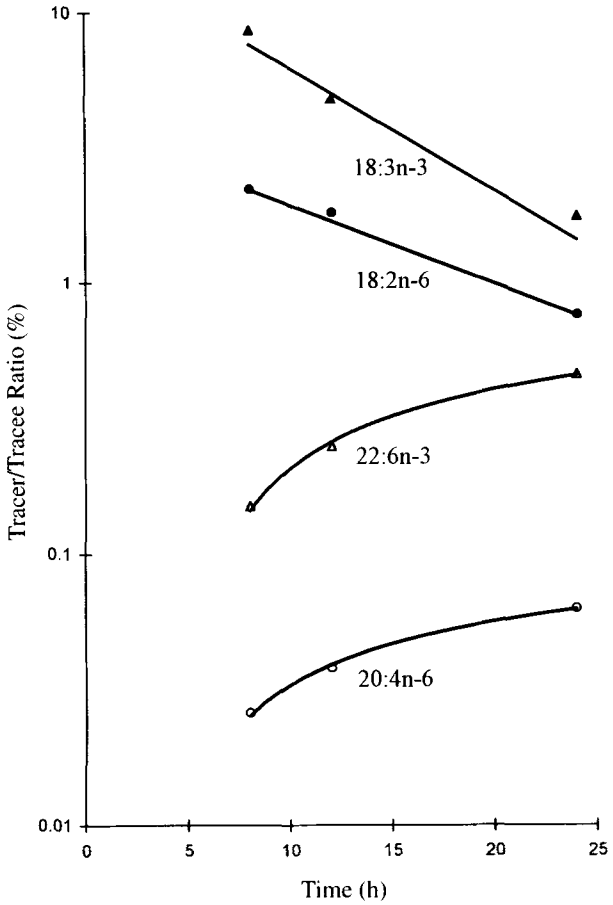


FIG. 2. Tracer/tracee ratios (specific activity) of precursors (18:3n-3 and 18:2n-6) and products (22:6n-3 and 20:4n-6) in plasma phospholipids of a representative three-week-old infant 8, 12, and 24 h after administration of [U-^{13}C]18:2n-6 and [U-^{13}C]18:3n-3 methyl esters (note semilogarithmic scale).

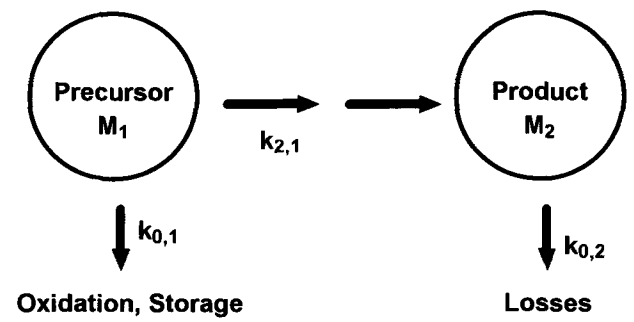


FIG. 1. Precursor/product compartmental model. M_1 and M_2 represent the plasma phospholipid precursor and product pools, respectively. Fractional catabolic rate of the precursor = $k_{0,1} + k_{2,1}$; fractional catabolic rate of the product = fractional rate of incorporation of the product = $k_{0,2}$; fractional conversion rate of the precursor = $k_{2,1}/(k_{0,1} + k_{2,1}) \times 100$.

cated, by a multiple comparison procedure (Fisher's Least Significant Difference).

RESULTS

Figure 2 shows the decline in enrichment of [M + 18] isotopomers of precursors, 18:3n-3 and 18:2n-6, and the increase in enrichment of [M + 18] isotopomers of 22:6n-3 and 20:4n-6 observed in a representative infant. Enrichments of [M + 18] isotopomers of n-3 fatty acids observed in the plasma phospholipid fraction of all infants 8, 12, and 24 h after administration of the labeled precursor are shown in Table 1. Those of the n-6 fatty acids at the same times are shown in Table 2. From these data, it is clear that all infants converted 18:3n-3 to 22:6n-3 and 18:2n-6 to 20:4n-6; however, there was considerable variability within as well as among groups. This variability is not surprising; the precursor enrichment is a function of the dose of precursor administered, the rate and extent of its absorption, as well as the rate and extent of its metabolism and incorporation into the plasma phospholipid fraction, all of which, in turn, affect enrichment of the products. Thus, to quantitate rates of conversion and incorporation, it is necessary to consider, simultaneously, enrichments of both precursors and products, including rates of change in enrichments of each.

TABLE 1
Isotopic Enrichments (% tracer/tracee) of n-3 Fatty Acids

	18:3n-3			22:6n-3		
	8 h	12 h	24 h	8 h	12 h	24 h
Group A						
1	28.18	19.88	4.41	0.09	0.15	0.27
2	4.95	9.95	3.43	0.02	0.05	0.10
3	18.61	12.26	3.34	0.03	0.06	0.14
4	23.48	15.75	4.20	0.29	0.43	0.96
5	50.23	22.37	6.80	0.06	0.12	0.24
6	39.31	26.49	6.51	0.09	0.18	0.40
Group B						
1	53.85	29.87	12.73	0.05	0.14	0.20
2	41.98	26.49	5.98	0.07	0.11	0.36
3	24.57	23.81	7.82	0.05	0.11	0.24
4	19.34	18.58	6.56	n.d.[a]	0.29	0.72
5	26.41	17.03	n.d.	0.16	0.36	n.d.
6	29.65	12.24	2.76	0.10	0.19	0.43
7	13.55	12.51	2.58	0.04	0.13	0.23
8	14.97	10.42	9.62	0.15	0.34	0.28
9	13.19	52.87	3.70	0.17	0.28	0.40
10	26.90	22.91	5.95	0.06	0.16	0.29
11	11.29	9.47	1.99	0.06	0.13	0.25
Group C						
1	6.80	4.83	2.36	0.05	0.14	0.24
2	1.15	0.95	0.39	0.003	0.009	0.019
3	8.58	4.78	1.76	0.15	0.25	0.46
4	4.13	2.93	1.26	0.01	0.03	0.09
5	9.47	6.27	2.25	0.05	0.10	0.23
6	7.27	4.61	1.25	0.03	0.06	0.10
7	3.71	2.83	0.81	0.01	0.03	0.08
8	1.83	1.07	0.33	0.03	0.06	0.10
9	1.84	0.94	0.31	0.01	0.02	0.04
10	1.73	1.25	0.47	0.02	0.04	0.10

[a]n.d. = Not detected.

TABLE 2
Isotopic Enrichments (% tracer/tracee) of n-6 Fatty Acids

	18:3n-3			22:6n-3		
	8 h	12 h	24 h	8 h	12 h	24 h
Group A						
1	0.58	0.46	0.20	0.005	0.010	0.015
2	0.21	0.15	0.07	0.002	0.003	0.008
3	0.46	0.48	0.19	0.005	0.014	0.019
4	1.90	1.58	0.75	0.063	0.086	0.108
5	1.80	1.43	0.62	0.038	0.046	0.075
6	0.84	0.91	0.42	0.028	0.049	0.069
Group B						
1	2.66	1.67	1.09	0.017	0.031	0.037
2	3.41	1.63	0.72	0.022	0.030	0.070
3	1.74	1.43	0.63	0.042	n.d.[a]	n.d.
4	2.04	1.61	0.72	0.032	0.070	0.091
5	1.65	1.20	0.56	0.055	0.048	0.105
6	1.60	0.73	0.27	0.016	0.025	0.034
7	0.83	0.68	0.32	n.d.	0.018	0.030
8	0.84	0.98	0.69	0.012	0.042	0.025
9	2.01	1.38	0.62	0.041	0.051	0.057
10	2.00	2.54	1.37	0.033	0.071	0.097
11	1.44	1.32	0.57	0.028	0.046	0.071
Group C						
1	2.04	1.24	0.83	0.012	0.022	0.035
2	1.82	0.25	0.21	0.001	0.003	0.010
3	2.22	1.82	0.76	0.026	0.038	0.063
4	1.57	1.36	0.74	0.005	0.010	0.023
5	3.48	2.64	1.17	0.024	0.039	0.062
6	1.38	1.24	0.57	0.009	0.014	0.020
7	0.45	0.47	0.21	0.002	0.004	0.010
8	0.63	0.47	0.19	0.013	0.021	0.028
9	0.45	0.43	0.17	0.003	0.007	0.012
10	0.34	0.35	0.17	n.d.	0.005	0.008

[a]n.d. = Not detected.

Mean rate constants and fractional rates of conversion and incorporation of the three groups, as calculated from the model using the data summarized in Tables 1 and 2, are shown in Table 3. Overall, the data from all infants fit the model with a residual error of 10% or less. The mean fractional rate of conversion of 18:3n-3 to 22:6n-3 did not differ significantly among groups. However, since the 18:3n-3 content of the plasma phospholipids (M_1) of group C was 3.7-fold larger than that of group A and 3.3-fold larger than that of group B, the fractional rate of incorporation of 22:6n-3 into the plasma phospholipid fraction was significantly higher in group C vs. groups A and B.

The mean fractional rate of conversion of 18:2n-6 to 20:4n-6 was lowest in group C, which received the highest 18:3n-3 intake. The mean fractional rate of incorporation of 20:4n-6 into the plasma phospholipid fraction also was lowest in group C.

DISCUSSION

The data reported above confirm that 3-wk-old term infants can convert 18:3n-3 and 18:2n-6, respectively, to 22:6n-3 and 20:4n-6. They also provide quantitative estimates of the effect of the dietary intake of 18:3n-3 on fractional rates of conversion of 18:3n-3 to 22:6n-3 and 18:2n-6 to 20:4n-6, as well as the fractional rates of incorporation of 22:6n-3 and 20:4n-6

TABLE 3
Parameters (mean ± SD) of the Precursor/Product Compartmental Model

	Group A 0.4% 18:3n-3 (n = 6)	Group B 1.0% 18:3n-3 (n = 11)	Group C 3.2% 18:3n-3 (n = 10)
n-3 Fatty acids			
M_1 (nmol/mL)	3.7 ± 1.2[a]	4.1 ± 1.4[a]	13.7 ± 1.8[b]
M_2 (nmol/mL)	41.5 ± 18.0[a]	64.7 ± 24.6[b]	78.9 ± 35.2[b]
$k_{0,1}$	2.48 ± 0.40[a]	2.33 ± 0.64[a,b]	1.96 ± 0.39[b]
$k_{2,1}$	0.18 ± 0.13[a]	0.32 ± 0.18[b]	0.21 ± 0.10[a,b]
FRC (% M_1/d)[c]	6.92 ± 5.18	12.13 ± 6.91	9.65 ± 3.86
FRI (% M_2/d)[d]	1.57 ± 1.54[a]	1.95 ± 1.01[a]	4.10 ± 2.18[b]
n-6 Fatty acids			
M_1 (nmol/mL)	770.7 ± 134.9[a]	1070.4 ± 90.9[b]	911.4 ± 76.6[a]
M_2 (nmol/mL)	205.4 ± 90.7	281.1 ± 55.8	219.0 ± 96.5
$k_{0,1}$	1.49 ± 0.09	1.63 ± 0.56	1.64 ± 0.60
$k_{2,1}$	0.017 ± 0.010[a]	0.012 ± 0.005[a]	0.006 ± 0.004[b]
FRC (% M_1/d)	1.13 ± 0.71[a]	0.82 ± 0.46[a]	0.41 ± 0.26[b]
FRI (% M_2/d)	5.94 ± 2.12[a]	4.42 ± 1.71[a]	2.66 ± 1.40[b]

[a,b]Values with different superscripts are significantly different ($P < 0.05$) from other values.
[c]FRC = Fractional rate of conversion $(k_{2,1}/(k_{0,1} + k_{2,1}) \times 100$.
[d]FRI = Fractional rate of incorporation $(k_{0,2} \times 100)$.

into the plasma phospholipid fraction. Dietary 18:3n-3 intake did not result in a statistically significant difference in fractional rates of conversion of 18:3n-3 to 22:6n-3. Because the 18:3n-3 content of the plasma phospholipid fraction was highest in the group that received the 3.2% 18:3n-3 intake, however, this group's fractional rate of incorporation of 22:6n-3 into the plasma phospholipid fraction was 2.5-fold greater than that of the group that received only 0.4% of total fat as 18:3n-3. In contrast, the highest vs. the lowest 18:3n-3 intake resulted in a 64% lower fractional rate of conversion of 18:2n-6 to 20:4n-6 and a more than 50% lower fractional rate of incorporation of 20:4n-6 into the plasma phospholipid fraction.

The validity of these conclusions, of course, depends on the validity of the model used to calculate fractional rates of conversion of precursor fatty acids and incorporation of the products into the plasma phospholipid fraction. In addition to the assumptions of steady state and equivalence of labeled and unlabeled pools, the model assumes that mass flow from precursor to product is unidirectional. While none of these assumptions is entirely valid, all are reasonable. More problematic with respect to the physiological relevance of the model are the assumptions that the only source of precursor converted to product is the precursor content of the plasma phospholipid fraction and that the only source of product incorporated into the plasma phospholipid fraction is that converted from the precursor content of this fraction. In addition, since it must be assumed that the plasma phospholipid fraction is part of a central, rapidly miscible pool, it also is assumed that the fatty acid composition of the plasma fraction is representative of all fractions of the central pool. Despite the number of assumptions inherent in the model, all apply equally to each dietary group. Thus, any differences among groups in calculated fractional rates of conversion of precursors to products and/or incorporation of products into the plasma phospholipid fraction are likely to reflect relative differences in fractional rates of conversion and incorporation incident to 18:3n-3 intake.

While the data reported apply strictly to only the plasma phospholipid fraction, it is likely that they are applicable to other fractions as well. For example, in rats, a modest increase in 18:3n-3 intake resulted in a lower 20:4n-6 content in all plasma and tissue lipid fractions (10). Further, in young human adult volunteers, a high intake of 18:2n-6 suppressed incorporation of long-chain polyunsaturated n-3 fatty acids into all plasma lipid fractions (11). Thus, it is very likely that the higher rate of incorporation of 22:6n-3 and the lower rate of incorporation of 20:4n-6 into plasma phospholipids observed in infants who received the highest dietary 18:3n-3 intake occurs also in the plasma triglyceride and cholesterol ester fractions. Interestingly, the mean fractional rate of incorporation of 20:4n-6 into the plasma phospholipid fraction observed in the group of infants who received the lowest 18:3n-3 intake (i.e., 5.9 ± 2.1% SD of the plasma phospholipid 20:4n-6 pool daily) is very similar to "the fractional rate of synthesis" of serum total lipid 20:4n-6 reported by Demmelmair *et al.* (1) in younger infants fed a low 18:3n-3 formula, i.e., 6.2 ± 1.0% SEM of the 20:4n-6 content of the serum total lipid fraction daily.

Unfortunately, neither the data reported here nor elsewhere are sufficient to calculate, or estimate, the amounts of long-chain polyunsaturated fatty acids synthesized daily. With respect to the data reported here, this is largely because there are no data concerning the magnitude of the pool of precursor and product fatty acids of any lipid fraction. This lack of information with respect to the plasma pool size can be remedied, but doing so will not clarify the relationship between plasma and tissue pools. Conversely, it is clear from the data reported here that a dietary 18:3n-3 intake of 3.2 vs. 0.4% of total fat results in a roughly 2.5-fold greater rate of incorporation of 22:6n-3 into the plasma phospholipid fraction and, concurrently, depresses both the rate of conversion of 18:2n-6 to 20:4n-6 and the rate of incorporation of 20:4n-6 into the plasma phospholipid fraction by about the same percentage. These are the first reported data concerning relative rates of conversion of 18:3n-3 and 18:2n-6 to 22:6n-3 and 20:4n-6, respectively, and the relative rates of incorporation of 22:6n-3 and 20:4n-6 into any lipid fraction. Whether these relative rates apply to fractions other than the plasma phospholipid fraction remains to be determined.

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Enzyme Replacement and Gene Therapy for Gaucher's Disease

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ABSTRACT: The lipid storage disorders have long been considered primary candidates for enzyme replacement therapy. This goal has been achieved with a remarkable degree of success in Gaucher's disease. Among the accomplishments that were important to obtain clinical benefit were the development of a large-scale procedure to purify human placental glucocerebrosidase and a method to target this enzyme to lipid-storing macrophages through glycoform modification. In addition, the effectiveness of recombinantly produced macrophage-targeted glucorerebrosidase has recently been demonstrated. Because macrophages originate from stem cells in the bone marrow, ex vivo transduction of these cells with retroviral vectors containing the cDNA for human glucocerebrosidase is being explored for the genetic therapy of Gaucher's disease.
Lipids 31, S-137–S-139 (1996).

Gaucher's disease is the most prevalent of the human lipid-storage disorders. It is caused by excessive accumulation of the sphingoglycolipid glucocerebroside (Fig. 1) in organs and tissues throughout the bodies of patients with this condition. The spleen and liver become greatly enlarged, pulmonary function decreases, and extensive deterioration of the skeleton frequently occurs. Patients are anemic and often have dangerously low platelets, predisposing them to hemorrhages. This disorder is caused by a deficiency of the enzyme glucocerebrosidase, which catalyzes hydrolytic cleavage of glucose from glucocerebroside (1). The product of the reaction, ceramide (*N*-fatty acyl sphingosine), is either reutilized for biosynthesis of larger sphingolipid homologues, such as sphingomyelin (ceramide-phosphocholine), or is further catabolized to sphingosine and free fatty acid. The latter reaction is catalyzed by the enyzme ceramidase. Because the activity of this enzyme in mammalian tissues is much less than that of glucocerebrosidase, it is presumed that reutilization of the ceramide is the preponderant pathway. Most of the glucocerebroside that accumulates in the spleen, liver, and bone marrow is believed to arise from the turnover of membrane components of red and white blood cells. The major neutral lipid in erythrocyte stroma is globoside (Fig. 2). Senescent red blood cells are removed from circulation primarily by macrophages in the spleen and liver. The stromal components are sequentially biodegraded by exoglycosidases. It is believed, however, that most of the glucocerebroside in these organs arises from the turnover of leukocytes (2), where lactosylceramide (Fig. 3) is the major sphingolipid component. In rare Gaucher patients with the infantile (type 2) phenotype with extensive brain involvement, glucocerebroside that accumulates in neurons is believed to be derived from gangliosides that turn over with great rapidity in the neonatal period of life.

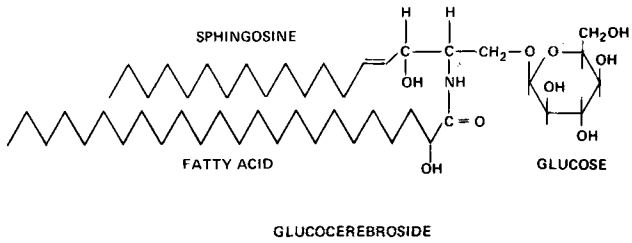


FIG. 1. Structure of glucocerebroside that accumulates in patients with Gaucher's disease.

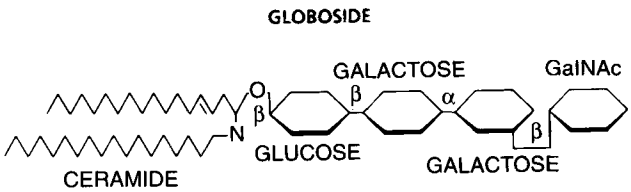


FIG. 2. Structure of globoside, the major neutral glycolipid of red blood cell stroma.

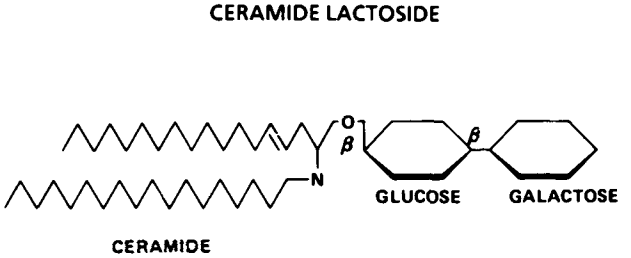


FIG. 3. Structure of lactosylceramide, the major neutral lipid of leukocytes.

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Enzyme replacement. Soon after the enzymatic defect in Gaucher's disease was demonstrated, both enzyme and gene (DNA) therapy were proposed for treatment of patients with this disorder (3). To explore the feasibility of enyzme therapy, human placental tissue was used as a source of glucocerebrosidase to minimize the sensitization of patients to an exogenous protein. After a long period of endeavor, a small amount of glucocerebrosidase was isolated that was of sufficient enough purity so that it would not be harmful if injected into patients with Gaucher's disease (4). We discovered that intravenous administration of placental glucocerebrosidase caused a significant reduction in the amount of glucocerebroside that had accumulated in the liver and lowered the concentration of this lipid in the blood from a three- to fourfold elevation to the normal range (5). Of particular note was the fact that the rate of reaccumulation of glucocerebroside, at least in the blood, was quite slow (6). We were not able to scaleup the original enzyme purification method to extend this investigation to more patients. We therefore spent a considerable amount of time developing another isolation procedure based on the high degree of hydrophobicity of glucocerebrosidase (7). An inconsistent reduction of hepatic glucocerebroside, however, was obtained when this preparation was administered to patients.

Most of our data indicates that intravenous infusion of exogenous glucocerebrosidase could reduce accumulated glucocerebroside. We believe that the lack of consistency seen with glucocerebrosidase purified by the hydrophobic chromatography procedure was due to lack of uniform delivery of this enzyme to macrophages where the lipid is stored. This inconsistency was eventually overcome in the following fashion. It was learned that lysosomal enzymes are glycoproteins (8) and that the major portion of glucocerebrosidase resides in these organelles (9). Macrophages were found to have a lectin on their surface that avidly interacts with mannose-terminal glycoconjugates (10). We analyzed the carbohydrate composition of glucocerebrosidase and found four oligosaccharide chains. Three of these exhibited a complex carbohydrate pattern terminating for the most part with *N*-acetylneuraminic acid (11). Mannose was present, but it was internal to molecules of *N*-acetylneuraminic acid, galactose, and *N*-acetylglucosamine (Fig. 4). Several procedures were carried out to enrich the mannose content of glucocerebrosidase (12). The most effective method was the sequential enzymatic cleavage of the three external monosaccharides with exoglycosidases to produce mannose-terminal enzyme. This preparation was much more effectively targeted to macrophages than native glucocerebrosidase (13).

We then began a series of investigations in patients with the macrophage-targeted glucocerebrosidase. We obtained convincing evidence of clinical benefit in a child with Gaucher's disease (14). We then performed a dose-response investigation to determine the quantity of enzyme that caused consistent reduction of stored glucocerebroside in patients (13). Based on the findings in this study, we elected to administer 60 U of macrophage-targeted glucocerebrosidase per kg of body weight every 2 weeks to patients with Gaucher's disease. Twelve patients participated in this clinical efficacy trial. All showed dramatic improvement in their clinical manifestations of Gaucher's disease (Fig. 5) (15). Their hemoglobin levels and platelet counts increased, the size of their enlarged spleens and livers greatly diminished, and impaired pulmonary function and skeletal abnormalities improved. On the basis of these findings, enzyme replacement therapy for patients with Gaucher's disease using macrophage-targeted human placental glucocerebrosidase was approved by the U.S. Food and Drug Administration on April 5, 1991. More than 1,500 patients throughout the world are now benefiting from this treatment.

Glucocerebrosidase was subsequently produced recombinantly in Chinese hamster ovary cells. This enzyme is also targeted to macrophages by the same exoglycosidase procedure described previously. Recombinant glucocerebrosidase is as effective as the placental enzyme for treatment of patients with Gaucher's disease (16). It is expected that this product will eventually completely supplant the use of placental enzyme.

Gene therapy. Because glucocerebroside-storing macrophages arise from stem cells in bone marrow, patients with Gaucher's disease are particularly attractive candidates for gene therapy. Retroviral vectors are commonly used to intro-

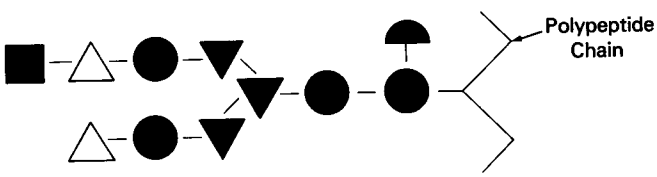


FIG 4. Principal oligosaccharide pattern of human placental glucocerebrosidase: ■ = *N*-acetylneuraminic acid; △ = galactose; ● = *N*-acetylglucosamine; ▼ = mannose; ◓ = fucose.

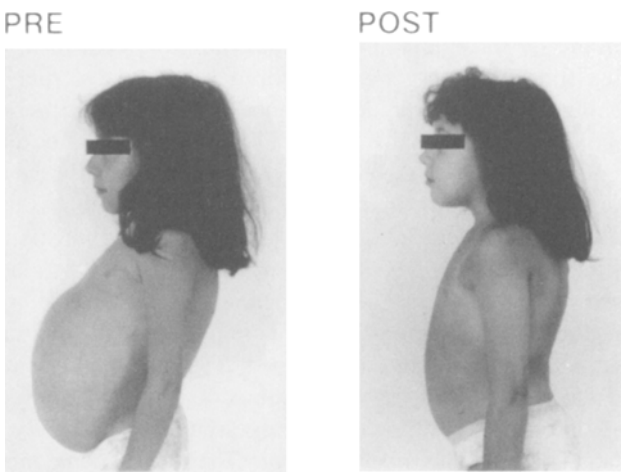


FIG. 5. Reduction of hepatosplenomegaly in a 10-year-old female with Gaucher's disease following enzyme replacement therapy for 24 mon.

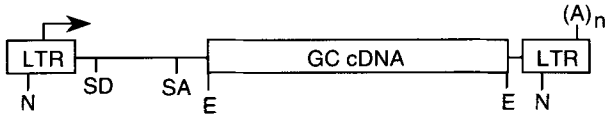


FIG. 6. Retrovial vector construct containing human glucocerebrosidase cDNA used to transduce murine, simian and human bone marrow stem cells. LTR = Moloney murine leukemia virus long-term repeat; $(A)_n$ = polyadenylation signal; N and E are sites for restriction endonucleases Nhe1 and EcoR1; GC is the complementary DNA for human glucocerebrosidase.

duce desired genes into dividing stem and progenitor cells. We therefore developed a construct containing the cDNA for human glucocerebrosidase (Fig. 6). We have shown that transduction of progenitor cells obtained from bone marrow of patients with Gaucher's disease restores glucocerebrosidase activity to a normal level in these cells (17). Long-term expression of human glucocerebrosidase has been documented in mouse macrophages (18). Recently, long-term expression of human glucocerebrosidase has also been demonstrated in nonhuman primates (19). These findings provide a firm basis for gene therapy for patients with Gaucher's disease. National Institutes of Health (NIH) Recombinant DNA Advisory Committee and the U.S. Food and Drug Administration have approved a phase-1 study of retroviral-mediated transfer of human glucocerebrosidase cDNA into hematopoietic stem cells of patients with Gaucher's disease. Both bone marrow and peripheral blood will be used as sources of stem cells. This investigation is expected to be begin promptly. It is anticipated that gene therapy will eventually be the treatment of choice for a number of lipid-storage diseases since, if effective, it will provide a permanent cure for patients with these disorders.

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Very Long-Chain Fatty Acids in Diagnosis, Pathogenesis, and Therapy of Peroxisomal Disorders

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ABSTRACT: Abnormally high levels of very long-chain fatty acids (VLCFA) are a feature in nine of the fifteen peroxisomal disorders that have been identified so far. Saturated VLCFA accumulate in X-linked adrenoleukodystrophy, appear to disrupt membrane structure, and may play a role in the pathogenesis of a brain inflammatory response. Dietary therapy initiated when patients are still asymptomatic may be of clinical benefit.
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Fifteen peroxisomal disorders have now been identified and are listed in Table 1. They are subdivided into two major groups, namely disorders of peroxisome assembly, in which the organelle fails to form normally and multiple peroxisomal functions are defective, and a second category in which defects involve a single peroxisomal protein and structure of the organelle is intact. Peroxisomal proteins are synthesized in free polyribosomes most commonly in their mature form, enter the cytoplasm, and are targeted to the organelle by mechanisms that are beginning to be clarified (1). Complementation analysis (2) indicates that there are at least 11 distinct genetic defects that can disturb these import mechanisms, and mutations have been defined in two of these (2). Ten of these complementation groups are associated with phenotypes that range from the Zellweger syndrome, the most severe form in which psychomotor development is virtually absent and usually leads to death during the first year, to the somewhat milder neonatal adrenoleukodystrophy (NALD) and infantile Refsum disease (IRD), in which the clinical manifestations resemble the Zellweger syndrome, but are somewhat milder, and some patients have reached adulthood. Most patients with the phenotype of rhizomelic chondrodysplasia punctata (RCDP) belong to a single and separate complementation group. X-Linked adrenoleukodystrophy (X-ALD) is the most common peroxisomal disorder. This gene defect involves a peroxisomal membrane protein that appears to be a member of the ATP-binding cassette transporter superfamily (3). Clinical manifestations associated with isolated deficiencies of acyl-CoA oxidase (AOX), bifunctional enzyme (BIF), or thiolase are indistinguishable from those of the Zellweger-NALD-IRD complex (2).

Levels of very long-chain fatty acids (VLCFA) in plasma are increased in the Zellweger syndrome, NALD, IRD, X-ALD, AOX, BIF, and peroxisomal thiolase deficiency, and our laboratory has diagnosed more than 3,000 patients with these disorders. The various disorders can be distinguished by combined appraisal of clinical features, biochemical patterns, and complementation analysis (2). The various disorders have somewhat different patterns of VLCFA excess (Table 2). Additional tests of peroxisomal function are often required for precise diagnosis. VLCFA levels are not increased in RCDP.

There is evidence that VLCFA play a pathogenetic role in several peroxisomal disorders. Pathological manifestations of X-ALD involve mainly the adrenal cortex and the nervous system. While adrenal dysfunction is the result of accumulation of cholesterol esterified with VLCFA, pathogenesis of the nervous system pathology is more complex. Two types of nervous system pathology occur (4). The first type involves part of the axon most distant from the cell body and is associated with a loss of myelin in tracts associated with these axons. It presents clinically as slowly progressive spasticity of the lower

Table 1
Peroxisomal Disorders

Assembly deficiencies	Single peroxisomal enzyme deficiencies
Zellweger syndrome	X-linked adrenoleukodystrophy
NALD[a]	Acyl-CoA oxidase deficiency
Infantile Refsum	Bifunctional enzyme deficiency
Hyperpipecolic acid	Peroxisomal thiolase deficiency
Rhizomelic chondrodysplasia punctata	DHAP[b] alkyl transferase deficiency
	Alkyl DHAP synthase deficiency
	Glutaric aciduria type III
	Classical Refsum disease
	Hyperoxaluria type 1
	Acatalasemia

[a]NALD = Neonatal adrenoleukodystrophy.
[b]DHAP = dihydroxyacetone phosphate.

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Abbreviations: AMN, adrenomyeloneuropathy; AOX, acyl-CoA oxidase; BIF, bifunctional enzyme; DHA, docosahexaenoic acid; IRD, infantile Refsum disease; NALD, neonatal adrenoleukodystrophy; RDCP, rhizomelic chondrodysplasia punctata; VLCFA, very long-chain fatty acids; X-ALD, X-linked adrenoleukodystrophy.

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extremities in the young adult, progresses slowly over decades, and is referred to as adrenomyeloneuropathy (AMN). In the second type, there is rapidly progressive demyelination, which affects mainly parieto-occipital lobes of the brain and spares the axons at least initially. There is intensive perivascular infiltration by T-cells, B-cells, and macrophages and prominent immunoreactivity for cytokines such as tumor necrosis factor in astrocytes and macrophages (4). This type of pathology occurs mainly in young boys and progresses rapidly, often leading to an apparently vegetative state within two years (5). These two highly variant phenotypes often occur within the same family. Segregation analysis suggests action of an autosomal modifier gene (5), which is thought to act by modulating intensity of the brain inflammatory response. Tumor necrosis factor α bioactivity is increased in serum of boys with the rapidly progressive form of X-ALD, but not in patients with AMN (6).

Excess of saturated VLCFA in membranes of X-ALD patients increases microviscosity of red cell membranes (7) and impairs capacity of cultured adrenal cells to respond to adrenocorticotrophic hormone (8). Physical properties of saturated VLCFA, such as hexacosanoic acid (C26:0), differ profoundly from palmitic and stearic acids. Albumin binds only one mole of C26:0, and rate of transfer of C26:0 from model membranes to albumin is 3 to 4 orders slower than that of palmitic acid. Calorimetric studies indicate that excess C26:0 disrupts membrane structure (9). Gradually increasing accumulation of saturated VLCFA may thus cause axonopathy in AMN. Powers *et al.* (4) postulate that in boys with brain inflammatory response, VLCFA excess leads to an additional and different pathological response, namely initiation of a cytokine-mediated inflammatory cascade.

Recent studies in our laboratory suggest that markedly increased levels of VLCFA also play a role in pathogenesis of neurological deficits in the Zellweger syndrome and disorders of peroxisome assembly. Zellweger syndrome patients have a severe and characteristic defect of neuronal migration (10), which is the major cause of the profound mental retardation and intractable seizures associated with these disorders. Because of the multiplicity of biochemical defects that exist in the Zellweger syndrome, it is difficult to pinpoint which of these are the cause of this neuropathological deficit. We have shown recently that a similar neuronal migrational defect existed in a patient with an isolated defect of the peroxisomal BIF (11). Correlational analysis suggests that these neuronal migrational defects occur in patients with plasma levels of C26:0 in excess of 3 μ/ml and suggest that the excess of VLCFA alone plays a role in pathogenesis of the neuronal migration defect (11). An entirely different additional neuropathogenetic mechanism is indicated by the studies of Martinez *et al.* (12), who demonstrated that patients with disorders of peroxisome assembly have severe deficiencies of docosahexaenoic acid. We have confirmed their findings in the study of plasma of 55 patients with various disorders of peroxisome assembly (Table 2).

Studies to determine whether reduction of VLCFA levels is of clinical benefit to patients with peroxisomal disorders are in progress. In patients with X-ALD, administration of a 4:1 mixture of glyceryl trioleate and glyceryl trierucate oil, also referred to as Lorenzo's Oil, combined with a reduced dietary fat intake, normalizes levels of saturated VLCFA in plasma within 4 wk (13). The oil is thought to act by reducing endogenous synthesis of saturated VLCFA by the microsomal elongating system. Administration of the oil also increased the plasma levels of docosahexaenoic acid (DHA) (Table 2). Clinical results have been disappointing in patients who already had neurological symptoms at the time therapy was begun (14). This lack of success in symptomatic patients may be due to the difficulty of reversing neurological damage that has already occurred, as has been the experience with phenylketonuria and congenital hypothyroidism.

Another possible explanation is the recent demonstration that, while considerable amounts of erucic acid were present in the postmortem liver and adipose tissues of patients who had been treated with the oils, levels of this substance in the brain were the same as in untreated patients (15). Administration of the oil thus may not have reduced the endogenous synthesis of saturated VLCFA in brain. Results have been more encouraging in 52 boys with X-ALD in whom dietary therapy was begun while they were still free of symptoms and who have continued on this therapy for a mean of 56 mon. Ten percent of these boys have become disabled (16) as compared to our estimate that this would have occurred in 30–40% of untreated patients. While these results are preliminary, because of the need for longer follow-up and statistical concerns, they are sufficiently encouraging to warrant continuation of the trial, and an international study protocol was initiated at the 1994 meeting of the Society for the Study of Inborn Errors of Metabolism. Diet must be monitored, since it causes a moderate reduction of platelet count in 40% of patients (17), which appears to be correlated with levels of erucic acid in platelets (18). Platelet count increases again when erucic acid intake is eliminated or reduced. Dietary therapy may cause a reduction of levels of DHA in plasma and red blood cells (Table 2), which can usually be restored by supplementation with essential fatty acids. We have also conducted studies of oral administration of DHA in five patients with disorders of peroxisome assembly. Biochemical results of this therapy confirm those presented by Martinez *et al.* (12).

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TABLE 2
Plasma Total Lipid Fatty Acids (μg/mL) in Patients with Peroxisomal Disorders

Fatty acid	Normal fasting (n = 56)	X-ALD[a]-Pre diet (n = 37)	ALD–Lorenzo's Oil (n = 59)	Mean STD Zellweger (n = 23)	NALD/IRD[b] (n = 34)	Bifunctional (n = 8)	RCDP[c] (n = 9)
14:0	9.99 ± 3.59	10.0 ± 6.54	7.5 ± 3.57	12.1 ± 7.09	12.4 ± 11.9	15.6 ± 6.96	12.0 ± 6.42
16:0	331 ± 73.0	287 ± 97.3	263 ± 66.0[e]	281 ± 104	267 ± 101[e]	318 ± 23.2	299 ± 57.8
PHYT[d]	0.61 ± 0.63			0.39 ± 0.25	11.1 ± 35.9	0.24 ± 0.13	2.02 ± 2.96
18:0	140 ± 19.9	132 ± 40.3	116 ± 24.4[e]	107 ± 38.3[e]	108 ± 45.1[e]	149 ± 34.4	131 ± 30.8
20:0	6.48 ± 1.28	3.7 ± 1.27[e]	3.23 ± 0.99[e]	4.09 ± 1.18[e]	3.46 ± 1.35[e]	6.14 ± 1.35[e]	6.63 ± 1.47
22:0	21.85 ± 4.86	12.1 ± 5.48[e]	6.81 ± 2.34[e]	5.61 ± 1.84[e]	5.75 ± 2.65[e]	9.72 ± 4.29[e]	11.8 ± 2.6[e]
24:0	17.85 ± 4.12	18.7 ± 8.36	8.78 ± 3.51[e]	11.0 ± 3.17[e]	8.08 ± 3.69[e]	18.18 ± 5.0	10.4 ± 2.57[e]
25:0	0.56 ± 0.22	0.89 ± 0.72[e]	0.97 ± 1.14	1.39 ± 0.91[e]	0.91 ± 0.63[e]	1.39 ± 0.87	0.42 ± 0.29
26:0	0.17 ± 0.05	0.68 ± 0.33[e]	0.45 ± 0.17[e]	2.71 ± 1.07[e]	1.39 ± 1.02[e]	3.14 ± 0.9[e]	0.26 ± 0.12
16:1n-7	26.2 ± 11.0	25.9 ± 18.3	23.1 ± 13.0	25.6 ± 16.5	22.8 ± 17.9	24.2 ± 12.8	29.8 ± 14.8
18:1n-7	38.7 ± 17.6	63.3 ± 38.6[e]	36.0 ± 23.3	10.6 ± 12.7[e]	17.1 ± 19.4	8.56 ± 12.5	26.5 ± 14.8
18:1n-9	288 ± 63.9	303 ± 131	417 ± 85.8[e]	204 ± 104[e]	257 ± 112	277 ± 97.2	284 ± 147
20:3n-9	1.89 ± 0.58	2.46 ± 1.99	3.62 ± 3.97[e]	2.84 ± 2.77	2.95 ± 2.90	5.87 ± 7.98	1.96 ± 1.32
22:1n-9	0.61 ± 0.20	0.63 ± 0.53	12.9 ± 9.35[e]	0.45 ± 0.22[e]	0.92 ± 1.42	0.41 ± 0.23[e]	0.73 ± 0.35
24:1n-9	20 ± 5.81	15.2 ± 8.51	40.9 ± 12.5[e]	15.9 ± 5.24[e]	16.1 ± 8.33	22.7 ± 4.95	22.2 ± 441
18:2n-6	434 ± 96.2	357 ± 117[e]	284 ± 68.8[e]	248 ± 129[e]	274 ± 137[e]	418 ± 177	320 ± 144
20:2n-6	0.98 ± 2.88	3.7 ± 1.41[e]	3.4 ± 0.89[e]	5.08 ± 2.93[e]	5.1 ± 3.44[e]	9.7 ± 5.51[e]	6.2 ± 3.13[e]
20:3n-6	23.98 ± 6.47	23.3 ± 8.51	19.9 ± 5.17[e]	13.0 ± 5.72[e]	15.5 ± 6.61[e]	29.5 ± 8.69	19.4 ± 7.89
20:4n-6	93.4 ± 26.0	80.5 ± 26.1	61.4 ± 19.2[e]	40.6 ± 13.5[e]	43.1 ± 19.3[e]	56.0 ± 16.0[e]	78.1 ± 19.5
22:4n-6	5.02 ± 1.10	5.34 ± 2.85	5.74 ± 3.08	2.73 ± 1.99[e]	2.81 ± 1.77[e]	3.07 ± 2.2	2.68 ± 1.38[e]
18:3n-3	5.79 ± 3.53	4.7 ± 3.18	4.71 ± 2.44	2.04 ± 1.8[e]	2.5 ± 1.23[e]	3.71 ± 2.02	3.31 ± 2.87
20:5n-3	3.05 ± 1.02	2.79 ± 2.18	2.58 ± 1.57	1.51 ± 1.0[e]	1.58 ± 1.05[e]	2.14 ± 0.88	2.21 ± 1.01
22:5n-3	3.65 ± 1.00	4.26 ± 1.79[e]	2.35 ± 1.09[e]	4.01 ± 1.4	4.35 ± 2.58	6.31 ± 3.27	3.65 ± 0.92
22:6n-3	19.0 ± 6.44	13.7 ± 7.79[e]	19.2 ± 8.72[e]	3.22 ± 2.93[e]	5.94 ± 6.13[e]	6.93 ± 6.93[e]	11.71 ± 4.82[e]
Totals							
Saturates	529 ± 107	465 ± 160	407 ± 102[e]	425 ± 146[e]	417 ± 146[e]	521 ± 51	473 ± 92
n-7	64.9 ± 28.6	89.2 ± 57	59 ± 36.2	36.2 ± 29.1	38.35 ± 27.2	32.7 ± 33	50.5 ± 28.5
n-9	310 ± 70.5	322 ± 141	474 ± 112[e]	223 ± 107[e]	277 ± 119	306 ± 94	309 ± 148
n-6	557 ± 133	470 ± 156	374 ± 97.2[e]	309 ± 139[e]	335 ± 154[e]	516 ± 184	427 ± 155
n-3	31.5 ± 12.0	25.5 ± 14.9[e]	28.8 ± 13.8	10.8 ± 3.8[e]	13.8 ± 7.52[e]	19.1 ± 5.2[e]	20.9 ± 6.2[e]
Total fatty acid	1492 ± 351	1372 ± 529	1344 ± 361	1002 ± 348[e]	1081 ± 355[e]	1644 ± 277	1280 ± 295

[a]ALD = Adrenoleukodystrophy.
[b]IRD = Infantile Refsum disease.
[c]RCDP = Rhizomelic chrondodysplasia punctata.
[d]PHYT = Phytanic acid.
[e]*P* Value ≤ 0.05.

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Losses of Arachidonic Acid in Rat Liver After Alcohol Inhalation

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ABSTRACT: This paper presents an animal model of alcoholism in which rats were exposed to alcohol by inhalation and were fed a diet that simulated the poor diet of some alcoholics. It is hypothesized that some of the pathophysiological effects of alcohol are related to its effects on essential fatty acid metabolism and composition of vital organs. A diet that contains no 20- and 22-carbon essential fatty acids and has low levels of 18-carbon essential fatty acids was used as a dietary challenge. Addition of a second metabolic challenge, i.e., alcohol, led to loss of tissue polyunsaturates, particularly liver arachidonate. A method of cycling alcohol inhalation for 12 h/d was also presented, which was also shown to lower liver arachidonic acid content.

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Alcoholism is a devastating illness of modern civilization that causes great misery, disease, and economic loss. It also is associated with homicide, assault, and suicide and affects 20–40 % of those hospitalized in urban settings (1). It is believed to affect nearly 14 million Americans. Confounding the direct toxic effects of alcohol on various organ systems is the poor diet that many alcoholics are believed to consume (2). It has been noted in recent reviews that alcohol exposure leads to the losses of essential fatty acids (EFA) in various tissues (3,4). It is hypothesized that losses of EFA may underlie some of the pathologic changes associated with alcoholism, particularly in the liver and brain. These losses may be the result of the direct and indirect effects of alcohol within the organism and a reduction in EFA intake due to poor dietary habits and food selection. In one recent publication, Pawlosky and Salem (5) demonstrated a loss in liver, plasma, and brain long-chain polyunsaturates in cats exposed to moderate levels of alcohol for 6 mo when they were fed a diet low in EFA (5).

A rodent model that employs a diet low in EFA together with alcohol exposure might be of great utility for the study of the potentiating effects of diet limitation of these nutrients on the course of alcoholic pathology. Changes in brain composition, in particular, have been somewhat variable in animal models of alcoholism (4), and a key uncontrolled variable may be the dietary EFA composition. This paper describes an attempt to develop such a rodent model.

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Abbreviation: EFA, essential fatty acids.

EXPERIMENTAL PROCEDURES

Animals and dietary treatment. Male Sprague–Dawley rats (70–80 g) were fed *ad libitum* for 4 wk on a powdered (Teklad, Madison, WI) 10 wt% fat diet containing 1 wt% corn oil and 9 wt% olive oil. The diet composition is presented in Table 1. At the end of the third week, they were divided in a

TABLE 1
Diet Composition[a]

Component	g/kg Diet
Vitamin-free casein	214.00
DL-methionine	3.20
Corn starch	426.90
Sucrose	150.00
Corn oil	10.00
Alphacel nonnutritive bulk	53.50
AIN-76 mineral mix	37.40
Calcium carbonate	4.30
Olive oil	90.00
Tenox GT-1	0.50
Kodak E567	0.07
Tenox 20A	0.10
Vitamin mix	
p-Aminobenzoic acid (PABA)	0.1200
Ascorbic acid	1.0900
Biotin	0.0005
Vitamin B_{12} (0.1% trit.)	0.0300
D calcium pantothenate	0.0700
Choline dihydrogen citrate	3.7400
Folic acid	0.0020
Inositol	0.1200
Menadione	0.0500
Niacin	0.1100
Pyridoxine HCl	0.0200
Riboflavin	0.0200
Thiamine HCl	0.0200
Vitamin A palmitate (250,000 IU/g)	0.0800
Vitamin D_3 (400,000 IU/g)	0.0060
Vitamin E acetate (500 IU/g)	0.2600
Corn starch	4.9900

[a]Diet from Teklad (Madison, WI).

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TABLE 2
The Effects of Alcohol Inhalation on Rat Liver Total Lipid Extract Fatty Acyl Composition of Rats Fed an Olive Oil-Based Diet[a]

Fatty acid	Control	Ethanol	*P* value[b]
Nonessential			
16:0	22.0 ± 0.4	23.2 ± 1.4	n.s.[c]
16:1n-7	2.8 ± 0.3	7.6 ± 1.1	0.001
18:0	10.3 ± 0.7	5.3 ± 0.6	0.0002
18:1n-9	29.7 ± 2.4	34.1 ± 2.4	n.s.
18:1n-7	4.4 ± 0.4	4.7 ± 0.5	n.s.
n-6 PUFA			
18:2	9.1 ± 0.3	8.1 ± 1.1	n.s.
18:3	0.1 ± 0.01	0.2 ± 0.05	n.s.
20:3	0.6 ± 0.09	0.6 ± 0.08	n.s.
20:4	12.4 ± 1.2	7.0 ± 1.2	0.005
22:4	0.3 ± 0.02	0.4 ± 0.06	n.s.
22:5	0.8 ± 0.07	0.4 ± 0.1	0.016
n-3 PUFA			
20:5	0.05 ± 0.01	0.08 ± 0.01	n.s.
22:5	0.2 ± 0.02	0.2 ± 0.03	n.s.
22:6	2.7 ± 0.2	2.0 ± 0.4	n.s.

[a]Data are expressed as the mean of the weight percent of each fatty acid ± SEM in the liver total lipid extract for n = 6 animals per group.
[b]Results were analyzed using unpaired, two-tail *t*-test. *P* values are indicated where less than 0.05.
[c]N.S., not significant.
[d]PUFA, polyunsaturated fatty acids.

TABLE 3
The Effects of Alcohol Inhalation on Rat Red Blood Cell Total Lipid Extract Fatty Acyl Composition of Rats Fed an Olive Oil-Based Diet[a]

Fatty acid	Control	Ethanol	*P* value[b]
Nonessential			
16:0	19.8 ± 0.7	19.0 ± 0.2	n.s.
18:0	12.5 ± 0.2	11.8 ± 0.3	0.041
18:1n-9	10.2 ± 0.4	10.3 ± 0.4	n.s.
18:1n-7	2.6 ± 0.1	2.9 ± 0.1	n.s.
n-6 PUFA			
18:2	6.1 ± 0.2	6.5 ± 0.2	n.s.
20:3	0.5 ± 0.01	0.7 ± 0.08	0.013
20:4	25.0 ± 0.4	25.1 ± 0.4	n.s.
22:4	1.9 ± 0.07	1.9 ± 0.03	n.s.
22:5	1.0 ± 0.05	0.9 ± 0.05	n.s.
n-3 PUFA			
20:5	0.2 ± 0.01	0.3 ± 0.02	n.s.
22:5	1.5 ± 0.04	1.7 ± 0.09	n.s.
22:6	3.3 ± 0.08	3.8 ± 0.1	0.002

[a]Data are expressed as the mean of the weight percent of each fatty acid ± SEM in the red blood cell total lipid extract for n = 6 animals per group. Abbreviations are as in Table 2.
[b]Results were analyzed using unpaired, two-tail *t*-test. *P* values are indicated where less than 0.05.

TABLE 4
The Effects of Alcohol Inhalation on Rat Platelet Total Lipid Extract Fatty Acyl Composition of Rats Fed an Olive Oil-Based Diet[a]

Fatty acid	Control	Ethanol	*P* value[b]
Nonessential			
16:0	8.2 ± 1.3	8.9 ± 0.9	n.s.
18:0	14.8 ± 1.6	13.8 ± 1.4	n.s.
18:1n-9	12.8 ± 0.7	13.7 ± 1.6	n.s.
18:1n-7	3.2 ± 0.09	3.8 ± 0.2	0.008
n-6 PUFA			
18:2	5.4 ± 0.2	5.5 ± 0.5	n.s.
20:3	0.6 ± 0.04	1.0 ± 0.2	n.s.
20:4	22.3 ± 1.8	20.2 ± 2.4	n.s.
22:4	3.7 ± 0.4	4.0 ± 0.5	n.s.
22:5	0.6 ± 0.06	0.5 ± 0.04	n.s.
n-3 PUFA			
18:3	0.09 ± 0.03	0.07 ± 0.02	n.s.
20:5	0.08 ± 0.01	0.1 ± 0.04	n.s.
22:5	0.3 ± 0.06	0.3 ± 0.07	n.s.
22:6	1.0 ± 0.2	0.8 ± 0.07	n.s.

[a]Data are expressed as the mean of the weight percent of each fatty acid ± SEM in the platelet total lipid extract for n = 6 animals per group. Abbreviations are as in Table 2.
[b]Results were analyzed using unpaired, two-tail *t*-test. *P* values are indicated where less than 0.05.

TABLE 5
The Effects of Alcohol Inhalation on Rat Brain Total Lipid Extract Fatty Acyl Composition of Rats Fed an Olive Oil-Based Diet[a]

Fatty acid	Control	Ethanol	*P* value[b]
Nonessential			
16:0	16.1 ± 0.2	15.3 ± 0.3	0.032
18:0	18.8 ± 0.1	18.5 ± 0.08	n.s.
18:1n-9	14.7 ± 0.2	14.8 ± 0.07	n.s.
18:1n-7	3.2 ± 0.05	3.3 ± 0.02	n.s.
n-6 PUFA			
18:2	0.5 ± 0.01	0.5 ± 0.01	n.s.
20:3	0.4 ± 0.01	0.5 ± 0.01	n.s.
20:4	10.8 ± 0.1	10.6 ± 0.06	n.s.
22:4	3.5 ± 0.05	3.6 ± 0.06	n.s.
22:5	0.80 ± 0.02	0.72 ± 0.03	0.032
n-3 PUFA			
22:5	0.14 ± 0.004	0.15 ± 0.005	n.s.
22:6	15.2 ± 0.2	15.4 ± 0.2	n.s.

[a]Data are expressed as the mean of the weight percent of each fatty acid ± SEM in the brain total lipid extract for n = 6 animals per group. Abbreviations are as in Table 2.
[b]Results were analyzed using unpaired, two-tail *t*-test. *P* values are indicated where less than 0.05.

random manner into two groups: half received 1 wk of continuous ethanol inhalation, and the other half were placed in inhalation chambers to which no alcohol was introduced. The design and implementation of the alcohol inhalation chambers was previously described in detail (6). Blood alcohol concentrations were determined after tail vein bleeding and the use of a colorimetric assay kit (Sigma Chemicals, St. Louis, MO). The rats were sacrificed at the end of the dietary treatments by cervical dislocation. All animal procedures were approved by the National Institute on Alcohol Abuse and Alcoholism Animal Care and Use Committee.

Fatty acid analysis. The rats were decapitated and organs removed for fatty acid analysis. The brain and approximately

1 g liver were removed and homogenized. Blood was removed and separated into red blood cells, platelets, and plasma by centrifugation. Lipids were extracted from all tissues using the method of Bligh and Dyer (7), with the exception that red blood cell lipids were extracted using the method of Reed *et al.* (8). A portion of the total lipid extracts from each tissue was transmethylated using 14% wt/vol BF_3-methanol at 100°C for 120 min to which hexane was added [25% vol/vol hexane, modified from Morrison and Smith (9)]. The fatty acid methyl esters were injected into a Hewlett Packard 5880A gas chromatograph (Palo Alto, CA) in the split mode with a DB-FFAP (J&W, LaPalma, CA) capillary column (0.25 mm i.d. x 30 m), a flame-ionization detector, and hydrogen as carrier gas at a linear velocity of 55 cm/sec. Injector and detector temperatures were set to 240°C, and the oven temperature program was as follows: 130°C to 175°C at 4°C/min, 175°C to 210°C at 1°C/min, 210°C to 240°C at 30°C/min, with a final hold of 30 min. The fatty acid methyl esters were identified by comparison of retention times to standard mixtures (Nu-Chek-Prep, Elysian, MN).

Statistical analysis. Data are expressed as the mean value (n = 6) plus or minus the standard error of the mean. A student's *t*-test (unpaired, two-tailed) was applied for data analysis between the alcohol and control groups. A $P < 0.05$ was considered significant.

RESULTS

Animals were fed a diet that included only the low levels of EFA present in 1 wt% corn oil plus those in olive oil (9 wt%) for 4 wk. These animals consumed an adequate, but rather low, level of linoleic acid (18:2n-6) on the order of 1.3 en%. A much lower supply of n-3 fatty acids in the form of linolenate (18:3n-3) was present, as it comprises only about 1 and 0.5% of corn and olive oil fatty acids, respectively. One group of these was then exposed to continuous alcohol inhalation during the last week, resulting in a group mean blood alcohol concentration of 180 mg% as measured in tail blood samples. Alterations in the fatty acyl composition of the liver, platelet, erythrocyte, and brain total lipid extracts were then assessed.

Alterations in the acyl composition of the liver was the most pronounced of the tissues and cell types analyzed (Table 2). There was a highly significant loss of arachidonic acid (20:4n-6) and docosapentaenoic acid (22:5n-6) in the liver with compensatory increases in nonessential fatty acids, especially 16:1n-7. There was a loss of 26% of the docosahexaenoic acid (22:6n-3) after alcohol exposure, but this did not reach statistical significance. In the erythrocyte, there were significant increases in 20:3n-6 and 22:6n-3, indicative of alterations in fatty acid metabolism (Table 3). In the platelet, losses in 20:4n-6 and 22:6n-3 did not reach significance; however, there were the characteristic increases in nonessential fatty acids such as 18:1 (Table 4). As is characteristic of the brain, there were few alterations of acyl composition after this brief alcohol exposure, with only the slight losses of 22:5n-6 and 16:0 reaching significance (Table 5). In several cases (liver, erythrocyte, and brain), there were significant losses in the saturates 16:0 or 18:0.

To further refine this model to be a more physiologically relevant one to human alcoholism, alcohol exposure was limited to 12 h/d. In this experiment, it was observed that the decrease in the 20:4n-6 level was approximately the same (Table 6) as during continuous exposure. After 24 h of alcohol withdrawal, there was some recovery in the 20:4n-6 level (Table 6).

DISCUSSION

It is striking that a 44% loss of liver 20:4n-6 could be achieved with only a 7 d exposure to alcohol. This fatty acid has many known functions, and so such a drastic reduction in its level would be expected to have negative consequences. This loss was mainly localized to cholesterol esters and phosphatidylcholines, and little or no losses were found in triglycerides or phosphatidylethanolamines. The magnitude of this loss induced by alcohol may have been due, in part, to the reductions in dietary EFA. There was no preformed 20:4n-6 available in the diet, and the level of its precursor, 18:2n-6, was also relatively low. It is hypothesized that this dietary state mimics that of many alcoholics and that the lack of dietary oversupply creates a permissive state in which alcohol can produce greater depletions of organ long-chain polyunsaturates and organ damage.

There were few changes in EFA in blood cells or the brain after 7 d of alcohol exposure, although human alcoholics

TABLE 6
Effects of 28 Days of Ethanol Inhalation Cycling on Rat Liver Total Lipid Extract Fatty Acyl Composition[a]

Fatty acid[b]	Control	Cycled ETOH	Withdrawal 24 h
Nonessential			
16:0	18.5 ± 0.3	19.7 ± 0.2[c]	18.7 ± 0.3
16:1n-7	1.4 ± 0.1	2.8 ± 0.6[c]	2.4 ± 0.4[c]
18:0	12.2 ± 0.3	9.3 ± 0.6[c]	10.4 ± 0.6[c]
18:1n-9	8.4 ± 0.3	13.5 ± 0.5[c]	12.5 ± 0.3[c]
18:1n-7	4.1 ± 0.2	3.8 ± 0.3	3.7 ± 0.1
n-6 PUFA			
18:2	21.0 ± 0.5	21.5 ± 0.9	21.7 ± 0.4
18:3	0.1 ± 0.01	0.2 ± 0.03[c]	0.2 ± 0.03
20:2	0.6 ± 0.02	0.4 ± 0.02[c]	0.3 ± 0.01[c]
20:3	1.5 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
20:4	12.9 ± 0.5	8.5 ± 0.3[c]	9.3 ± 0.4[c]
22:4	0.2 ± 0.01	0.4 ± 0.1	0.3 ± 0.02[c]
22:5	0.08 ± 0.004	0.1 ± 0.01[c]	0.1 ± 0.008[c]
n-3 PUFA			
18:3	0.8 ± 0.04	1.3 ± 0.1[c]	1.2 ± 0.1[c]
20:5	2.0 ± 0.05	1.5 ± 0.1[c]	1.6 ± 0.1
22:5	3.0 ± 0.05	2.2 ± 0.1[c]	2.4 ± 0.1[c]
22:6	7.7 ± 0.3	9.2 ± 0.4[c]	9.5 ± 0.3[c]

[a]Animals were fed an NIH-07 chow diet and exposed to ethanol vapors for 12 h per day for 28 d prior to sacrifice.
[b]Data are expressed as the mean ± SEM weight percent for two experiments of n = 5 animals each.
[c]Experimental groups were compared to controls by an unpaired two-tailed *t*-test. Significant differences are noted with $P < 0.05$.

often exhibit such alterations (3,4). This may be attributed principally to the relatively short period of alcohol exposure in this experiment. For example, domestic cats subjected to a single daily dose of alcohol for 6 mo exhibited drastic losses in most plasma and liver EFA, while nonessential fatty acids in the liver increased markedly (5). It was also important that this study showed a significant loss of brain and retinal 22:6n-3, despite the resistance of this fatty acid to nutritional challenge (10–12). It thus appears that a more sustained alcohol exposure in rats will be necessary.

This study also employed a diet where the EFA were supplied by 1 wt% corn oil, and so was relatively low in these nutrients. It should be noted that these diets will by themselves lead to lower tissue levels of tissue long-chain (C_{20} and C_{22}) polyunsaturated fatty acids. This effect, however, is reflected in the dietary control values, and it is clear that alcohol exposure led to a further, greater diminution of these fatty acids.

Improvement in the rodent model of human alcoholism was also achieved with the use of cycling alcohol exposure limited to 12 h/d. This allowed the mucous membranes of the animals to recover and provided for more normal activity and eating behavior. Future experiments will further define EFA levels in the diet as modulatory variables for ethanol-related pathological effects with more prolonged exposure periods.

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Arachidonic Acid to Eicosapentaenoic Acid Ratio in Blood Correlates Positively with Clinical Symptoms of Depression

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ABSTRACT: In this study of 20 moderately to severely depressed patients, diagnosed using current research diagnostic criteria and excluding known bipolar affective disorder and reactive depression, we investigated relationships between severity of depression and levels and ratios of n-3 and n-6 long-chain polyunsaturated fatty acids (PUFA) in plasma and erythrocyte phospholipids (PL). Severity of depression was measured using the 21-item Hamilton depression rating scale (HRS) and a second linear rating scale (LRS) of severity of depressive symptoms that omitted anxiety symptoms. There was a significant correlation between the ratio of erythrocyte PL arachidonic acid (AA) to eicosapentaenoic acid (EPA) and severity of depression as rated by the HRS ($P < 0.05$) and the LRS for depression ($P < 0.01$). There was also a significant negative correlation between erythrocyte EPA and the LRS ($P < 0.05$). The AA/EPA ratio in plasma PL and the ratio of erythrocyte long-chain (C_{20} and C_{22} carbon) n-6 to long-chain n-3 PUFA were also significantly correlated with the LRS ($P < 0.05$). These findings do not appear to be simply explained by differences in dietary intake of EPA. We cannot determine whether the high ratios of AA/EPA in both plasma and erythrocyte PL are the result of depression or whether tissue PUFA change predate the depressive symptoms. We suggest, however, that our findings provide a basis for studying the effect of the nutritional supplementation of depressed subjects, aimed at reducing the AA/EPA ratio in tissues and severity of depression.
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Major depression is occurring at a younger age and at a higher incidence than it had been prior to 1913 (1,2). In epidemiological studies reviewed by Klerman (1), these findings, primarily for Westernized societies, were not explained by changing diagnostic criteria, changing attitudes of health professionals and societies, reporting bias, differential mortality, or institutional or other artifacts.

Of the many changes in Western society, the changes in diet corresponding to population increase in the industrial period of 1850–1995 is considered to have contributed to development of a range of medical conditions, especially cardiovascular disease and inflammatory disorders (3,4). The increase in total fats, saturated fats, and linoleic acid (LA)-rich vegetable oils during this time has been associated with a large increase in n-6/n-3 PUFA in the diet (4,5). The n-6/n-3 PUFA ratio is estimated to have risen from about 1:1 to greater than 10:1 as fats from fish, wild game, and leaves were replaced by increasing consumption of LA-rich oils from seeds. This dietary imbalance of n-6/n-3 PUFA leads to a high proportion of arachidonic acid (AA) and little eicosapentaenoic acid (EPA) in the cell membranes of most tissues, resulting in an overproduction of eicosanoids derived from n-6 PUFA such as prostacyclin, thromboxane, leukotrienes (3), and inflammatory mediators (cytokines and interleukins) (6).

Epidemiological evidence from the Multiple Risk Factor Intervention Trial of 12,866 middle-aged men in the United States revealed that there were significant inverse associations between dietary n-3 PUFA [α-linolenic acid (ALA) and long-chain n-3 PUFA] and mortality from coronary heart disease, all cardiovascular mortality, and all-cause mortality (7). In addition, there were negative associations between the ratio of n-3/n-6 PUFA (ALA/LA and total n-3/n-6 PUFA) and cancer mortality.

Smith (8) and Hibbeln and Salem (9) have hypothesized that the sharp rises in depression and other neurological disorders in this century have been fueled by increased consumption of LA-rich vegetable oils. Consistent with this hypothesis are the substantially elevated levels of prostaglandin E_2 and thromboxane B_2 (both derived from AA, a metabolite of LA) reported in patients with both unipolar and bipolar depression (10).

In this study, we investigated relationships between severity of depression and levels and ratios of n-3 and n-6 long-chain PUFA in plasma and erythrocyte PL of 20 moderately to severely depressed patients, diagnosed using research diagnostic criteria (11).

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; HRS, Hamilton rating scale; LA, linoleic acid; LRS, linear rating scale; PL, phospholipids; PUFA, polyunsaturated fatty acids.

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MATERIALS AND METHODS

Patients were selected from those consecutively referred for routine psychiatric treatment who were either inpatients or outpatients of the participating psychiatrists (P.A. and S.L.). Criteria for participating in the study was a diagnosis of unipolar depression according to the criteria in DSM IV (11), with no past or present diagnosis of bipolar affective disorder. There were no age or sex exclusion criteria, and in one case a second psychiatric diagnosis (anorexia nervosa) was present (Table 1). At initial assessment, all but three patients were drug-free: patient 9 was taking 50 mg doxepin, patient 13 had just commenced dothiepin 150 mg at night, and patient 11 was taking low-dose digoxin and frusemide for cardiac failure. Protocol used for this study complied with the Helsinki Declaration of 1975 as revised in 1983.

Severity of depression was measured at commencement of the study and again after treatment using the 21-item Hamilton depression rating scale (HRS) (12), which defines mild (<17), moderate (17–34), and severe depressions (>34). This was assessed by direct interview by one of the two treating psychiatrists. The HRS includes both depressive and anxiety subscales, and for this reason a clinical rating of depression, omitting anxiety symptoms, was made using a predefined simple linear estimation of severity of depressive symptoms. This rating was from 0–10 and was based on averaging the clinical impression of the two participating psychiatrists after discussion of each case. Symptoms used were severity of mood disturbance, loss of appetite, weight loss, sleep disturbance, and suicidality. A score of 1–4 was rated as mild depression, 5–7 was moderate depression, and 8–10 was severe depression. The raters were both experienced clinical psychiatrists who previously standardized their ratings for depression over a 10-yr period.

TABLE 1
Clinical Details of Subjects in Study

Subject (number	Age (yr)	Sex	HRS score[a]	LRS score[b]	Fish intake (meals/wk)
1	50	M	30	9	1.5
2	21	F	21[c]	6	<0.5
3	42	M	24	6	2.0
4	38	M	24	6	1.0
5	79	M	36	10	0
6	33	F	30[c]	8	1.0
7	45	M	32	7	<0.5
8	38	M	30	6	0
9	44	F	24	7	0
10	55	M	27	7	1.5
11	77	M	19	6	1.0
12	46	F	20	7	0.5
13	29	F	18	5	0.5
14	29	F	24	7	0.9
15	78	F	23	7	<0.5
16	53	F	28	6	>3.5
17	19	F	27	8	0.9
18	59	F	26	8	0.5
19	72	M	14.5	8	1.5
20	23	F	21	8.5	1

[a]HRS range from 0–40; HRS = Hamilton rating scale.
[b]LRS range from 0–10; LRS = linear rating scale.
[c]These patients were diagnosed as suffering from anorexia nervosa.

At presentation, all patients completed an assessment of dietary intake of lipids over the previous three months, based on a food frequency questionnaire from Deakin University (Geelong, Australia) (13) and a diary of all food eaten the subsequent week to check for major changes over that period. The itemized assessment included frequency of intake of butter, margarine, milk products, vegetable oils, meat including liver and kidney, fresh and canned fish, eggs, nuts, olives, avocados, cakes, cookies, pastry products, and other snack foods. Intakes of AA and EPA were calculated from data from the food frequency questionnaire and use of a locally constructed database of the long-chain PUFA content of foods (14).

At the beginning of the study, prior to any change in medication, a 10-mL blood sample (in an EDTA tube) was taken from each patient after an overnight fast. The plasma was separated by centrifugation and stored at −20°C until analyzed. Erythrocytes were washed three times in 0.9% saline and then extracted in 10 mL of chloroform-methanol (1:1, containing 10 mg/L butylated hydroxytoluene) and stored at −20°C. Blood samples were coded and analyzed blind by the lipid analyst. Plasma and erythrocyte lipids were extracted in chloroform/methanol by standard procedures (15), PL were separated from total lipids by thin layer chromatography, and PL fatty acids were formed by saponification in KOH followed by methylation using boron trifluoride in methanol (15). Fatty acid methyl esters were separated by capillary gas–liquid chromatography, as previously described (15).

Standard, routine treatment for depression was prescribed by the two participating psychiatrists, according to clinical indications. In most cases, treatment was with tricyclic antidepressants with clinical review within two to five days, followed by weekly or biweekly reviews.

Results are expressed as means ± SD, and correlations were calculated using the Pearson correlation coefficient (r). A P value of <0.05 was considered significant. All statistical analyses were performed on Minitab Version 8 (Minitab, Inc., State College, PA).

RESULTS

Clinical findings. The age of the 20 patients ranged from 19 to 79 years. The HRS measurements for depression indicated one patient with severe depression (HRS score 36), and one with mild depression (HRS score 14.5), and the remainder were moderately depressed (HRS scores from 17–34). The linear rating scale (LRS) indicated more cases of severe depression than did the HRS, due to the previously described differences between the two scales. According to the LRS, 13 patients were classified as moderately depressed and 7 as severely depressed (Table 1).

Dietary findings Ten patients reported consuming 4–5 meat meals per week, 0–2 fish meals per week, 0.5–4

eggs/week, use of margarine/vegetable oils, and infrequent consumption of nuts. One patient had a similar diet, but consumed 12 eggs per week. Five patients reported a high meat intake (>7 meals per week); 0–1 fish meals per week; 2–10 eggs/week; butter, margarine, or vegetable oil use, and infrequent consumption of nuts. Of this group, three reported a frequent consumption of snack foods. Of the remaining four subjects, patients 2 and 6 were severely anorexic with a low intake of all nutrients; patient 9 was strictly vegetarian (no fish or eggs) and patient 16 reported a fish intake of between 3–4 meals per week, less than 4 meat meals per week, 5 eggs per week, use of vegetable oils, and consumption of nuts several times per week. No patients reported major changes in the major dietary contributors of n-6 and n-3 PUFA in the 3 months prior to presentation with depression.

Biochemical findings. Plasma PUFA values for the patients (Table 2) fell within the range we previously reported for 65 female and 43 male healthy normal Australian subjects (17).

As shown in Table 3, there were significant negative correlations between erythrocyte EPA levels and the LRS for depression ($r = -0.546$, $P < 0.05$) and plasma PL docosapentaenoic acid n-3 and the LRS for depression ($r = -0.539$, $P < 0.05$) (Table 3). There were significant positive correlations between the ratio of AA/EPA in the erythrocyte PL and plasma PL with the LRS ($r = +0.729$, $P < 0.01$, and +0.527, $P < 0.05$, respectively). The AA/EPA ratio in the erythrocyte PL was also correlated with the HRS ($r = +0.472$, $P < 0.05$) (Fig. 1). The erythrocyte long-chain PUFA n-6/long-chain PUFA n-3 ratio was also significantly correlated with the LRS ($r = +0.455$, $P < 0.05$).

Clinical findings. All patients showed significant improvement in depressive symptoms over 1 to 3 months and returned to normal living and occupational activities with HRS scores of less than 10 and LRS scores of less than 4. An exception was one frail elderly man (patient 5), admitted to the hospital with severe malnutrition, who died of myocardial infarction after making a modest improvement in mood with a low dose of the antidepressant medication, mianserin.

TABLE 2
Mean Plasma Phospholipid and Erythrocyte Phospholipid (PL) Polyunsaturated Fatty Acid Levels in Depressed Patients

Fatty acid	Patient plasma PL[a]	Patient erythrocyte PL[b]
n-6 PUFA[c]		
Linoleic acid	38.5 ± 11.9	12.4 ± 2.8
Eicosatrienoic acid	4.7 ± 2.1	1.7 ± 0.5
Arachidonic acid (AA)	18.9 ± 5.8	17.3 ± 1.8
Docosatetraenoic acid	0.5 ± 0.2	3.0 ± 0.5
Docosapentaenoic acid	0.5 ± 0.3	0.6 ± 0.2
Total n-6 PUFA	62.1 ± 16.4	35.0 ± 3.4
n-3 PUFA		
Eicosapentaenoic acid (EPA)	1.6 ± 0.6	0.8 ± 0.2
Docosapentaenoic acid	1.6 ± 0.5	2.8 ± 0.2
Docosahexaenoic acid	5.9 ± 2.1	4.7 ± 0.9
Total n-3 PUFA	9.2 ± 2.6	8.3 ± 1.0
n-6/n-3 PUFA	7.0 ± 1.6	4.3 ± 0.7
AA/EPA	12.4 ± 3.7	22.9 ± 5.3

[a]Results shown as mean ± SD, mg/100 mL plasma.
[b]Results shown as mean ± SD, percent of total fatty acids.
[c]PUFA = polyunsaturated fatty acids.

TABLE 3
Correlation Coefficient (*r*) Values Between Erythrocyte and Plasma Phospholipid PUFA Levels and Severity of Depression[a]

Polyunsaturated fatty acid	Hamilton rating scale	Linear rating scale
Erythrocyte PL[b] EPA	−0.290	−0.546[c]
Plasma PL EPA	+0.089	−0.416
Erythrocyte PL docosahexaenoic acid	+0.122	+0.100
Plasma PL docosahexaenoic acid	+0.001	+0.045
Erythrocyte PL docosapentaenoic acid n-3	−0.032	−0.224
Plasma PL docosapentaenoic acid n-3	−0.001	−0.539[c]
Erythrocyte PL AA/EPA	+0.472[c]	+0.729[d]
Plasma PL AA/EPA	+0.194	+0.527[c]
Erythrocyte PL long-chain n-6/long-chain n-3 PUFA	+0.006	+0.455[c]
Plasma PL long-chain n-6/long-chain n-3 PUFA	+0.310	+0.184

[a]Results are shown as the Pearson correlation coefficient (*r*).
[b]See Table 2 for abbreviations.
[c]$P < 0.05$
[d]$P < 0.01$.

DISCUSSION

Depression in Westernized countries is becoming increasingly common (1,2). Multifactorial hypotheses have been offered to account for depression, and diet is not thought to play a direct initial role. In some cases of depression, severe appetite loss, decreased food intake, and weight loss may occur, and these may contribute to changes in levels and/or proportions of dietary PUFA. These clinical features were found in most but not every patient in the study. Because n-6 PUFA are ubiquitous in the food supply and n-3 PUFA are relatively restricted (canola oil, fish, and other marine products), it is conceivable that appetite loss may be associated with selection of foods that are relatively low in n-3 PUFA. This was not a particular feature of this group, however,. Interpretation of the possible influences of nutrition on depression is confounded by the difficulty of determining whether depression antedates or predates dietary change.

The present group of subjects had plasma PUFA values within normal range for a larger group of healthy normal Australian subjects (17). The ratio of AA/EPA in the subjects' plasma PL and erythrocyte PL, however, correlated strongly with both the LRS and HRS. The ratio of the long-chain PUFA n-6/long-chain PUFA n-3 ratio in the erythrocyte PL was also correlated with severity of depression. The two parent PUFA in each family, LA and ALA, are in competition with each other for conversion to the long-chain 20- and 22-carbon PUFA. Diets that contain an increased level of n-6

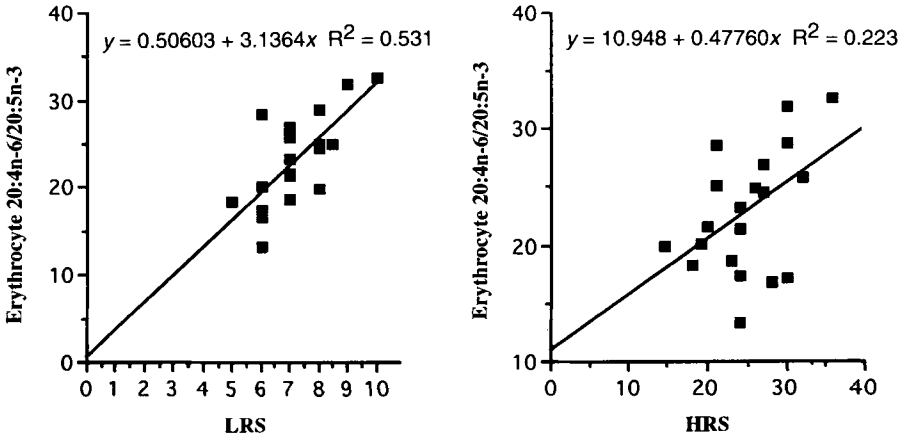


FIG. 1. The relationship between the erythrocyte 20:4n-6/20:5n-3 values and two ratings of depression (LRS = linear rating scale, $r = +0.729$, $P < 0.01$; HRS = Hamilton rating scale, $r = +0.472$, $P < 0.05$).

PUFA relative to n-3 PUFA lead to elevated levels of AA and low levels of EPA in plasma and erythrocyte lipids (18).

The concentration of PUFA in plasma PL has been used as a guide to the dietary intake of PUFA (18,19), although erythrocyte PUFA patterns have been more commonly used to indicate long-term dietary intake (20). Our findings do not appear to be simply explained by differences in intake of n-6 and n-3 PUFA between subjects, since intake of EPA, estimated from the food frequency questionnaire data, did not reveal any significant correlations with plasma or erythrocyte AA/EPA ratios ($r = 0.39$, $P > 0.05$ and $r = 0.10$, $P > 0.05$, respectively). Futhermore, patient 9 (vegetarian diet), patient 16 (high fish intake), and patient 2 (severe anorexia) had similar ratings for depression, despite major differences in diet and estimated EPA intakes (10, 380, and 50 mg per day, respectively).

Moderate to severe depression is often associated with sleep disturbance, markedly decreased food intake, and loss of weight (11). Since PUFA are essential fatty acids, changes in appetite could possibly lead to a reduced intake of both n-6 and n-3 PUFA or to an unbalanced intake of one PUFA type relative to the other. The Australian diet, like other Westernized diets, is rich in LA and relatively poor in ALA (21). LA is found in highest concentration in vegetable oils, and thus in PUFA-rich margarine, but it is also in a wide range of other foods (21). Lean meat, organ meats, and eggs contain LA and also small quantities of AA, which is readily incorporated into cell membranes (15). Normally, a low level of long-chain n-3 PUFA is the result of a low dietary intake of either the parent n-3 PUFA (ALA) from sources such as canola oil, soybean oil, and walnuts (21,22), or of the long-chain n-3 PUFA (EPA is found in highest concentration in fish and other marine foods such as shellfish, mollusks and the like, and fish oil). In this study, estimated intake of EPA from fish did not correlate significantly with plasma and erythrocyte levels of EPA ($r = 0.37$ and 0.35, respectively, $P > 0.05$), suggesting that diet was not the major influence on EPA levels in the subjects in this study.

We cannot determine whether the high ratios of AA/EPA in both plasma and erythrocyte PL are the result of depression or whether tissue PUFA changes predate the depressive symptoms. The association between the state of depression and erythrocyte and plasma PUFA levels may be a cause, an effect, or a reflection of other changes occurring during depression.

Results of this study contrast with reports from the United Kingdom by Ellis and Sanders (23) and Fehily *et al.* (24), who showed increases in EPA and DHA (long-chain n-3 PUFA) in plasma and erythrocyte PL in patients with severe depression. The patients in the present study were selected by research diagnostic criteria (11) and were a more homogeneous group than the two groups studied by Ellis and Sanders (23) and Fehily *et al.* (24), whose patients included those with known bipolar affective disorder and reactive or mild depression.

We have demonstrated a relationship between severity of depression and the plasma and erythrocyte AA/EPA ratio. More data is required before a link between dietary change, increased rates of depression and blood n-6/n-3 PUFA levels is established in a causative manner. We suggest that our findings provide a basis for studying the effect of nutritional supplementation of depressed subjects aimed at reducing the AA/EPA ratio in tissues and the severity of depression.

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Fatty Acids and Schizophrenia

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ABSTRACT: In a controlled study of red cell membrane fatty acids in patients with schizophrenia, substantial depletions of fatty acids from both the n-6 and n-3 series were demonstrated. Arachidonic acid and docosahexaenoic acid were particularly depleted. In a separate study, dietary analysis revealed no deficiency of fatty acid intake in this patient group, but greater intake of n-3 fatty acids was associated with less severe symptomatology. Dietary supplementation for six weeks with 10 g per day of concentrated fish oil (MaxEPA) led to significant improvement in schizophrenic symptoms. This clinical improvement was related to the increased level of n-3 fatty acids in red cell membranes. These findings form part of a growing body of research data suggestive of an abnormality in cell membrane fatty acid composition in schizophrenia. The preliminary evidence for clinically effective dietary manipulation to correct such an abnormality opens up novel and exciting therapeutic possibilities.
Lipids 31, S-163–S-165 (1996).

Schizophrenia is a serious mental disorder, the aetiology of which remains unknown. From the clinical perspective, signs and symptoms are many and varied and are often classified into "positive" and "negative" symptoms. Positive symptoms refer to the delusions, hallucinations, thought alienation, and thought disorder which typically occur early on in the course of the illness and which respond well to treatment with neuroleptic drugs. The negative symptoms include emotional blunting, social withdrawal, poverty of speech and activity and cognitive deficits. These tend to occur later in the course of the illness and respond poorly to neuroleptic treatment.

The aetiology of the condition remains uncertain. The theory that it results from increased dopaminergic activity in mesolimbic and mesocortical neuronal pathways has persisted for many years. However, more recently, research interest has focused on other neurotransmitter systems including glutamatergic and serotoninergic. There is evidence for a genetic component in the aetiology and, more recently, researchers have found accumulating indirect evidence to suggest that for a subgroup, at least, the disorder is neurodevelopmental in origin (1).

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Abbreviations: AA, Arachidonic acid; DHA, docosahexaenoic acid; TBARS, thiobarbituric acid reactive substances.

There is a small growing body of evidence indicating that phospholipid metabolism may be disturbed in schizophrenia. Interest has particularly begun to focus on abnormalities in the cell membrane fatty acid composition and the possible effects of such abnormalities on neuronal functioning.

Plasma fatty acids in schizophrenia. Over the last 20 y, various research workers have investigated levels of essential fatty acids in the plasma of schizophrenic patients. One early study demonstrated a raised level of α-linolenic acid (2), and work on schizophrenic patients from England, Scotland, and Ireland found consistently low levels of plasma linoleic acid and arachidonic acid (3). This latter group also demonstrated an increase in total n-3 fatty acids in plasma. Low levels of plasma linoleic acid were also found in a cohort of schizophrenic patients in Japan (4).

The most consistent reported finding is therefore the reduction of plasma linoleic acid. Similar results were found in twins, with twins concordant for schizophrenia showing greater abnormalities than those who were discordant (5).

Red Cell Membrane Fatty Acids in Schizophrenia. Our group in Sheffield has conducted a study of the fatty acid composition of red cell membranes from 23 drug treated schizophrenic patients and demonstrated substantial depletions of fatty acids from both the n-6 and n-3 series when compared to a healthy control group (Table 1). Arachidonic acid (AA) and docosahexaenoic acid (DHA) were particularly depleted. In addition, significant negative correlations between the depleted n-6 fatty acids and plasma levels of thiobarbituric acid reactive substances (TBARS), a marker of lipid peroxidation, suggested that the depletion was due to increased breakdown of the fatty acids rather than impaired incorporation of the fatty acids into membranes. Both AA and DHA concentrations appeared to be distributed bimodally. However, because measurements of symptomatology were not undertaken during this study, it was not possible to comment on any association between abnormal membrane fatty acids and symptomatology (6). In a similar study conducted in Scotland, symptoms were assessed using established rating scales. Patients with predominantly positive symptoms did not differ significantly from the control group whereas those with predominantly negative features of schizophrenia had significantly reduced levels of essential fatty acids, in partic-

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TABLE 1
Fatty Acid Composition of Red Cell Membranes from Schizophrenic Patients and Control Subjects[a]

	n-6 series		n-3 series	
	18:2n-6	20:4n-6	20:5n-3	22:6n-3
Schizophrenics	9.7[b]	11.9[c]	1.01	3.7[b]
(n = 23)	(0.6)	(6.2)	(0.4)	(2.4)
Controls	14.2	17.3	1.6	7.2
(n = 16)	(2.0)	(3.6)	(0.4)	(1.3)

[a]Expressed at mg/100 mg phospholipid, mean, and SD. Only those fatty acids showing significant differences are listed.
[b]$P < 0.001$ student's *t*-test.
[c]$P < 0.002$.

ular AA and DHA. The authors suggest from their data that there is a sub-population of schizophrenic patients with significantly reduced AA and DHA in red cell membranes but not in plasma and that these patients are highly likely to have predominantly negative symptoms (7).

These deficits in DHA and AA are of special interest as these fatty acids are particularly concentrated in brain tissue. There is evidence from primate work to suggest that red cell fatty acid concentrations may reflect those of neuronal tissue (8).

Evidence for increased lipid peroxidation. The work to date investigating plasma and red cell membrane fatty acids in schizophrenia raises intriguing questions regarding the possible role of abnormal fatty acid metabolism in the chemical pathology of schizophrenia. There is, in addition, an accumulating body of data indicating increased oxidative stress in this patient group.

Two studies have demonstrated increased lipid peroxidation in schizophrenia by their findings of increased pentane levels in expired air (9,10). This is consistent with our finding of a significant negative correlation between depleted n-6 fatty acids and plasma levels of TBARS which implies increased phospholipid breakdown rather than decreased incorporation of fatty acids into the red cell membrane (7). It is interesting in the light of these results to note that increased activity of the phospholipid metabolizing enzyme phospholipase A_2 has been demonstrated in a group of schizophrenic patients and that this activity has been shown to be reduced following neuroleptic treatment (11,12). Other workers have indicated that neuroleptic drugs can reduce the breakdown of phospholipids by inhibiting phospholipase A_2 (13). Consistent with this notion of enhanced lipid peroxidation in schizophrenia is the finding in a recent controlled study of increased plasma TBARS in 20 drug-naive patients during their first psychotic episodes (14). Increased TBARS was associated with lower red cell levels of the antioxidant enzyme glutathione peroxidase and a greater severity of negative symptoms at the onset of psychosis. The possibility of peroxodative damage of neuronal tissue leading to ventricular enlargement in schizophrenia is suggested by a reported correlation between depletion of glutathione peroxidase and increased ventricle-brain ratio (15). The finding of cerebral ventricular enlargement in some schizophrenics has previously been associated with negative symptoms (16).

Neuroimaging studies. Using magnetic resonance spectroscopy, decreased levels of phosphomonoesters and increased phosphodiesters have been demonstrated in the prefrontal cortex of drug naive schizophrenic patients (17). This is suggestive of decreased synthesis and/or increased breakdown of membrane phospholipids in schizophrenic patients. These findings have been confirmed by other workers (18) and there is also some evidence that this index of increased phospholipid breakdown correlates with severity of schizophrenic symptoms (19). This body of neuro-imaging data adds more credence to the hypothesis that there is an abnormality of membrane phospholipid fatty acid composition in patients with schizophrenia.

Supplementation studies. The importance of dietary factors in schizophrenia has not been paid a great deal of attention. In this respect it is of particular interest that outcome figures from the World Health Organization Schizophrenia Study have been shown to correlate with national dietary characteristics (20). Countries in which the national diet included relatively more essential fatty acids had a better national outcome of schizophrenia than those countries in which relatively more animal fat was consumed. In addition there is evidence that the fatty acid composition of neuronal membranes can be modified by dietary fatty acid intake (21,22). Attempts have been made to treat schizophrenia with n-6 essential fatty acids including γ-linolenic acid (23). These trials produced modest and variable improvements from the clinical perspective.

Until very recently, effects of n-3 fatty acid supplementation on schizophrenic symptoms have not been investigated. Our group in Sheffield therefore studied the effects of dietary supplementation with 10 g per day of concentrated fish oil (MaxEPA) over a six week period in a cohort of 20 schizophrenic patients (24). Supplementation resulted in significant increases of red cell membrane levels of eicosapentanoic acid as well as other n-3 fatty acids. There was a concomitant reduction in membrane n-6 fatty acids. Both positive and negative symptoms improved though only negative symptoms reached statistical significance. The improvement in clinical symptoms was related to the increased level of n-3 fatty acids in red cell membranes. In the course of this study, a detailed dietary analysis of fatty acid intake was carried out prior to the supplementation. None of the patients were deficient in fatty acid intake, but it was demonstrated that a greater intake of n-3 fatty acids and particularly eicosapentanoic acid was associated with less severe schizophrenic symptoms as well as less tardive dyskinesia (a movement disorder associated with prolonged neuroleptic drug treatment). In terms of any relationships between the red cell fatty acid levels prior to supplementation and schizophrenic symptomatology, both the total symptom score and severity of abnormal involuntary movements (tardive dyskinesia) showed significant positive correlations with n-6 fatty acids in the red cell membranes.

Conclusion. The findings presented in this paper from our own group and other workers represents a growing body of research data pointing towards an abnormality of phospholipid fatty acid metabolism in schizophrenia. Such an abnormality may be of aetiological significance. This possibility is not

incompatible with current receptor based theories as fatty acids are known to interact with the relevant receptors including those for dopamine and glutamate. Activation of dopamine D2 receptors enhances calcium dependent AA release *in vitro*, and this is reversed by haloperidol, a neuroleptic drug (25). AA has multiple effects on neuronal signal transduction, including acting as a second messenger in glutamatergic neurotransmission (26). It has also been demonstrated that membrane fatty acid composition is capable of modulating receptor function and neurotransmitter release including that of dopamine (27). We have previously hypothesised that several of the biological abnormalities reported in schizophrenic patients can be related to abnormal metabolism of AA (28).

The possibility that such an abnormality may be amenable to dietary manipulation opens up interesting therapeutic possibilities. The clinical improvements with the initial open study of n-3 fatty acid supplementation reported here are encouraging. Larger placebo controlled trials are required to clarify the efficacy of such an approach.

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Role of Essential Fatty Acids in the Function of the Developing Nervous System

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ABSTRACT: The basis for n-3 fatty acid essentiality in humans includes not only biochemical evidence but functional measures associated with n-3 deficiency in human and nonhuman primates. Functional development of the retina and the occipital cortex are affected by α-linolenic acid deficiency and by a lack of docosahexaenoic acid (DHA) in preterm infant formulas and, as reported more recently, in term diets. Functional effects of n-3 supply on sleep–wake cycles and heart rate rhythms support the need for dietary n-3 fatty acids during early development. Our results indicate that n-3 long-chain polyunsaturated fatty acids should be considered provisionally essential for infant nutrition. DHA may also be required by individuals with inherited metabolic defects in elongation and desaturation activity, such as patients with peroxisomal disorders and some forms of retinitis pigmentosa.

Lipids 31, S-167–S-176 (1996).

Dietary essential fatty acids (EFA) have been considered part of the lipid supply necessary for energy, growth, cellular metabolism, and muscle activity. The fact that some EFA serve as indispensable dietary precursors for eicosanoid (prostaglandins, prostacyclins, thromboxanes, leukotrienes) and docosanoid formation has given greater significance to their study. During the past decade, increasing attention has focused on the role of n-3 and n-6 EFA in normal fetal and neonatal development (1–7).

Basis for nutritional essentiality. The concept that specific components of fat may be necessary for the proper growth and development of animals and possibly humans was introduced in the 1930s (8). Yet, EFA were considered of marginal importance until the 1960s, when signs of clinical deficiency became apparent in infants fed skim milk–based formula and in those given lipid-free parental nutrition (9–12). During the past decade, it has become generally accepted that n-6 as well as n-3 fatty acids (FA) play a key role in perinatal nutrition, especially for the developing central nervous system (CNS).

Clinical EFA deficiency is associated with a set of specific findings (7,9–12). This syndrome has been related mostly to combined n-3 and n-6 deficits and includes poor growth; skin lesions; decreased skin pigmentation; loss of muscle tone; degenerative changes in kidney, lung, and liver; increased metabolic rate; impaired water balance; increased fragility and permeability of cell membranes; and increased susceptibility to infection (7,9–12).

Clinical manifestations disappear after administration of diets that provide 2% or more of the total calories as linoleic acid (LA, 18:2n-6) (9–14). More subtle clinical symptoms appear in n-3 EFA deficiency. They include skin changes unresponsive to LA supplementation, abnormal visual function, and peripheral neuropathy (15). Nervous system manifestations are likely caused by an insufficient supply of the metabolic derivative of α-linolenic acid (LNA, 18:3n-3), namely docosahexaenoic acid (DHA, 22:6n-3).

Structural role of EFA in the developing nervous system. Fetus and placenta are fully dependent on maternal EFA supply for growth and development. The mother's diet before pregnancy plays an important role in determining maternal EFA status, since these essential nutrients are stored in adipose tissue and can be mobilized over time through lipolysis. Major EFA deposition in the human fetus occurs during the third trimester; phospholipids in placental vessels and uterine vasculature are dependent on EFA supplied by the mother for eicosanoid formation. Maternal preconceptional nutrition determines, in part, which specific fats accumulate in the conceptus and placental tissues (6,16,17).

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Abbreviations: AA, arachidonic acid (20:4n-6) ; ADRP, autosomal dominant retinitis pigmentosa; AS, active sleep; CNS, central nervous system; DHA, docosahexaenoic acid (22:6n-3); DPA (n-6) and DPA, docosapentaenoic acid (22:5n-6 and 22:5n-3); EFA, essential fatty acids; EPA, eicosapentaenoic acid (20:5n-3) ; ERG, electroretinogram; FA, fatty acids; HM, human milk; HRV, heart rate variability; LA, linoleic acid (18:2n-6) ; LCPUFA, long-chain polyunsaturated fatty acids; LNA, α-linolenic acid (18:3n-3) ; MAR, minimal angle of resolution; QS, quiet sleep; RBC, red blood cells; REM, rapid eye movement; RP, retinitis pigmentosa; TPN, total parental nutrition; VEP, visual evoked potential; XLRP, X-linked retinitis pigmentosa.

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The dry weight of the human brain is predominantly lipid; 22% of the cerebral cortex and 24% of white matter consist of phospholipids. Studies of several animal species and recent evidence from humans established that brain phospholipid arachidonic acid (AA, 20:4n-6) and DHA decrease, while n-9 and n-7 mono- and polyunsaturated FA increase when LA and LNA or only n-3 FA are deficient in the diet (18–20). Typically, n-3 FA-deficient cells have decreased DHA and increased levels of the end products of n-3 and n-6 metabolism, DHA and docosapentaenoic acid (DPA, 22:5n-6), respectively. Within subcellular organelles, synaptosomes and mitochondria seem to be more sensitive to a low dietary n-3 supply (19–21).

High concentrations of long-chain derivatives of EFA in the cerebral cortex and retina suggests their participation in neural and visual function. The n-3 end product, DHA, reaches levels of up to 50% of total FA in the phospholipids of these tissues (22–24). A specific association of either DHA or AA with neural tissue function has not been fully defined. In the human, Clandinin and colleagues (24) showed significant increases in AA and DHA contents of brain tissue during the last trimester of gestation. More recently, Martinez (25) provided complementary data on retinal and brain accretion of long-chain polyunsaturated fatty acids (LCPUFA), using modern methodology.

The potential for dietary EFA deficiency has become a significant issue for the nutrition of preterm-born infants, since they do not receive the third-trimester intrauterine supply of DHA and AA. Even full-term infants are at risk of DHA deficiency since most formulas are devoid of this critical EFA. Recent postmortem investigations in term infants suggest a dependency on diet in the FA composition of brain cortex. Infants who are breast fed have higher DHA and lower DPA (n-6) and AA in their cortex phospholipids relative to infants receiving cow milk-based formulas (26, 27). DHA content increases with advancing age in breast-fed infants and is proportional to the duration of human-milk feeding, while AA content remains stable. Furthermore, the dietary ratio of n-6 to n-3 FA appears relevant, as the lowest DHA and highest DPA (n-6) contents in brain cortex phospholipids were observed in preterm infants fed formulas with a high LA to LNA ratio (27).

Biochemical and functional effects of EFA in the developing nervous system. Studies in nonhuman primates characterized biochemical and functional effects of dietary n-3 deficiency. Animal models were developed using purified LA as the only dietary fat or using safflower or sunflower oil in which the n-6/n-3 FA ratio (approximately 250:1) was very high (28–31). High n-6/n-3 FA ratios are found in powdered infant formulas currently in use in some parts of the world (32, 33). If these diets are fed from conception and/or during early life, significant declines in DHA levels and elevations of DPA (n-6) contents of brain, retina, and other tissues occur in animals. Similar data was obtained from plasma and red blood cell (RBC) lipids in preterm infants fed corn oil-based formulas that have a high LA/LNA ratio (34).

Use of RBC lipids as a potential index of EFA status in retinal membranes is based on correlations between FA profiles in RBC and neural/retinal tissue from rats and nonhuman primates and in human infants (27, 35, 36).

Effects of n-3 deficiency, specifically DHA, were demonstrated on retinal photoreceptor function and maturation of cortical visual function in nonhuman primates and in preterm infants. Improved cognitive function measured by psychometric tests

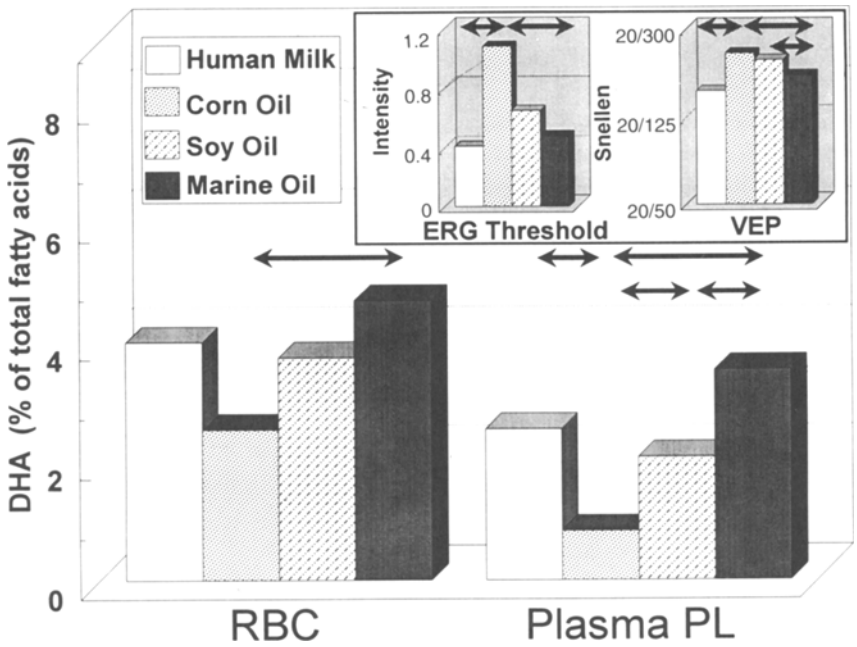


FIG. 1. Docosahexaenoic acid (DHA) in total red blood cell (RBC) lipids and plasma phospholipids (PL) of preterm infants on study diets at 36 wk postconception. Arrows indicate significant differences ($P < 0.05$) between diet groups using Newman–Keuls multiple comparison analysis: inset; rod electroretinogram (ERG) thresholds (intensity units = log scotopic troland seconds) and visual evoked potential (VEP) visual acuity (Snellen equivalents) in study infants at 36 wk.

were also shown in DHA-supplemented infants at 12 mon of age.

Infants fed fat-free parental diets provide nutritional models to study the effect of EFA deficiency on the developing brain. Initially, total parental nutrition (TPN) did not include lipids in its formulation. This allowed Fagioli *et al.* (37) to characterize effects of long-term dietary EFA deficiency on sleep organization. Studies in children who were maintained on fat-free TPN for 2 to 6 mon had consistently reduced durations of slow-wave sleep compared with children who received TPN plus essential lipids. The main difference was a lower cumulative amount of slow-wave sleep time from the second hour of night-sleep onward. These results suggest that early lipid nutrition may modulate sleep regulation and may have implications for whole-body energy expenditure during sleep, since slow-wave sleep is associated with a lower metabolic rate than are other stages of sleep (38). Interestingly, no significant difference was found in either total sleep time or intrasleep waking in the EFA-deficient group. Alterations in sleep–wake cycle development may become evident only if the methods used include evaluations that are more subtle than total sleep or waking time measures. The possibility that fat-free TPN affected structural brain lipids is suggested from these results (26, 27, 37).

Studies in preterm infants fed controlled EFA intake. Our studies (conducted in Dallas, Texas, and continued as a collaborative work in both Dallas and Santiago, Chile) have served to document the essentiality of n-3 FA in humans. Initial studies were performed in very-low-birth-weight infants, since they are particularly vulnerable to deficiency, given the virtual absence of adipose tissue stores at birth. This deficiency may be attributable to a possible immaturity of FA elongation/desaturation pathways and inadequate LNA and DHA intakes provided by formula. We and others conducted studies to evaluate the effect of n-3 in very low birth weight infants, examining effects of LNA or LNA plus DHA supplementation on plasma and tissue lipid composition, retinal electrophysiologic function, maturation of the visual cortex, and other measures of infant growth and development (39–45).

Before the 1990s, low levels of LNA were present in most infant formulas; virtually all infant formulas in developed countries are currently supplemented with LNA, and several manufacturers in Europe and Japan have added DHA or DHA plus AA to preterm formula (32, 33, 46). EFA composition of human milk (HM) and study formulas used in our studies were published previously (34). Briefly, corn oil-based formula corresponded to commercial, powdered premature formula, contained 24% LA, was extremely low in LNA (0.5%), and had no LCPUFA; soy oil-based formula was equivalent to present ready-to-feed premature formula used in the United States; and the third study formula was also soy oil-based (1.4% LNA) but was enriched with marine oil to give an n-3 LCPUFA content closer to that found in HM. At the time we started our study, n-3 LCPUFA could only be obtained from marine oil. We chose to include sufficient marine oil to mimic DHA content of HM, since we were uncertain as to how effectively preterm infants could convert eicosapentenoic acid (EPA, 20:5n-3) to DHA. The full nutritional content of the preterm milk formula and details of laboratory methods have been published (34, 39).

Composition of RBC lipids for all diet groups were similar on entry into the study (34). At the 36- and 57-wk postconception

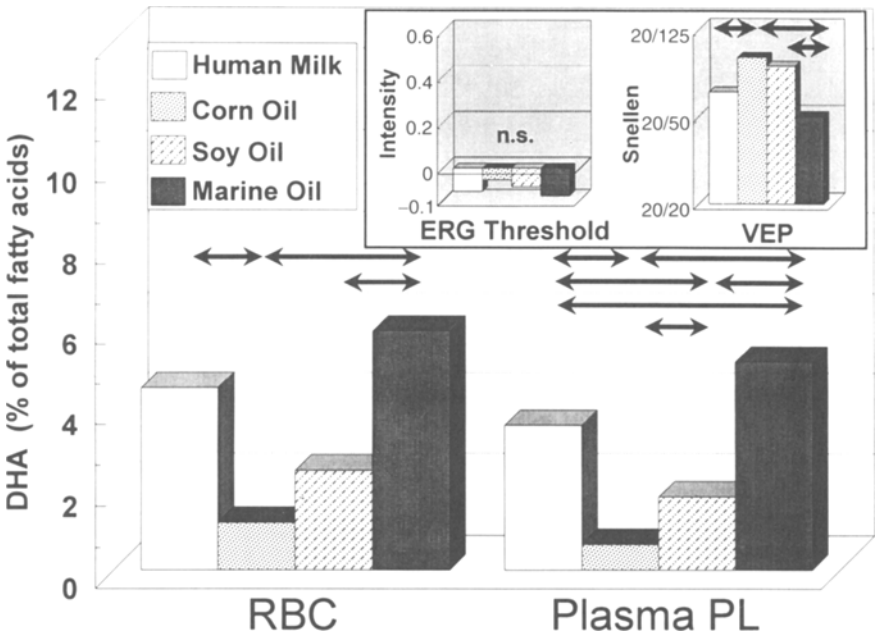


FIG. 2. Docosahexaenoic acid (DHA) in total red blood cell (RBC) lipids and plasma phospholipids (PL) of preterm infants on study diets at 57 wk postconception. Arrows indicate significant differences ($P < 0.05$) between diet groups using Newman–Keuls multiple comparison analysis: inset; rod electroretinogram (ERG) thresholds (intensity units = log scotopic troland seconds) and visual evoked potential (VEP) visual acuity (Snellen equivalents) in study infants at 57 wk.

follow-up, marked differences in DHA contents were evident in RBC lipids of all diet groups. Infants fed the n-3 FA-deficient corn oil-formula had significant reductions in DHA as compared to other formula groups. The marine oil group presented DHA concentrations elevated above all other groups. DPA (n-6) relative content found at 36 and 57 wk in RBC lipids was inversely related to DHA content. DPA (n-6) was significantly elevated in the corn oil group, in comparison to the HM and soy/marine groups. By 57 wk, differences in content of the endproducts of n-3 and n-6 FA metabolism [i.e., DHA and DPA (n-6), respectively] were greatly accentuated, compared to 36-wk values. The soy oil group had intermediate DHA and DPA (n-6) values relative to the corn and soy/marine oil groups but differed significantly from the DHA-supplemented infants (34). Figures 1 and 2 summarize changes in DHA content of RBC and plasma phospholipids observed at 36 and 57 wk postconception, respectively.

Methods for assessment of visual function have been described elsewhere, and detailed results of our studies have been recently published (39–41, 47–51). Electroretinographic responses demonstrated significantly higher threshold values from rod photoreceptors in the n-3–deficient group (corn oil-fed) at the 36-wk follow-up relative to groups receiving n-3 FA (26, 28), indicating that the sensitivity and maturity of rod photoreceptors in n-3–deficient infants were reduced significantly compared to infants fed soy/marine formula or HM. A group of 35-wk gestational age infants had their visual maturity tested a few days (3–5 d) after birth and served as a normal standard for comparison. This group of infants receiving transplacentally acquired EFA were nearly identical in all rod electroretinogram (ERG) functional indices to the equivalent conceptional age HM-fed or marine oil–fed premature infants. The soy oil-fed group had higher threshold values than did the soy/marine oil-fed group. Cone photoreceptor function was not significantly affected by diet, although trends were similar to results obtained from rods. At the 57-wk follow-up, corn oil-fed infants consistently had longer implicit times in light-adapted oscillatory potentials. The soy oil-fed infants had some response peaks differ significantly from the HM-fed infants, while the soy/marine and HM groups were similar in oscillatory potential responses. Oscillatory potentials are generated in the inner retina, mature later than distal retinal responses and most likely reflect ganglion cell–amacrine cell interactions (40).

The soy/marine oil- and the HM-fed groups had lower values of log MAR (minimal angle of resolution) (i.e., better acuity) relative to infants fed formula devoid of DHA at the 57-wk follow-up using either visual evoked potentials (VEP) or forced-choice preferential looking response methods. A log MAR value of 0 corresponds to 20/20 Snellen equivalents, while a value of 1 corresponds to 20/200. A significant negative correlation was found between RBC DHA levels and log MAR values; that is, when erythrocyte DHA levels were high, log MAR values were correspondingly low (i.e., better visual acuities). HM- and soy/marine oil–fed infants had the highest DHA/DPA (n-6) ratio in RBC and also had the best visual acuity, i.e., lowest log MAR values. The group that received soy oil as a source of n-3 had poorer VEP acuity at the 57-wk follow-up relative to the soy/marine oil-fed group, indicating that, despite ample LNA, visual function was less mature than in the DHA-supplemented infants. A group of healthy full-term, breast-fed infants matched by conceptional age was used as a control. HM- and soy/marine oil-fed groups were virtually identical to the control group, while corn and soy groups had poorer visual acuities (41, 48). Insets of Figures 1 and 2 summarize changes in ERG threshold and in VEP acuity observed in the four diet groups at 36 and 57 wk postconception, respectively. Visual acuity at 4 mon in full-term HM-fed infants was significantly better than in formula-fed infants and was virtually identical to the HM- and marine oil-fed premature groups.

Reversibility of changes in visual function observed in preterm infants cannot be fully answered from our data since the study was terminated at 57 wk postconception. Most of the ERG alterations at 36 wk normalized with time, based on the 57-wk results, except for the ganglion cell–dependent, oscillatory potentials.

Other functional effects are being evaluated in our present studies in premature infants fed HM or one of three formulas (control, lacking LCPUFA; supplemented with AA and DHA; or supplemented solely with DHA). These measures include organization of sleep–wake cycles during early development, changes in heart rate patterns during sleep states, and spontaneous activity detected by piezoelectric accelerometers during various sleep states and the wakeful period. At the time of this report, the study remains masked; thus results comparing LCPUFA-supplemented groups vs. the unsupplemented group have not been made, but comparisons between formula- and breast-fed groups are revealing.

Since intrauterine life is characterized by the virtual absence of wake periods (52) and since postnatal infants spend most of their time sleeping, study of sleep–wake cycles is an important component in evaluation of CNS development. Specific developmental patterns in infants have been described, and long-term effects of changes in sleep–wake cycles have been documented.

Sleep–wake cycle has been well characterized as a function of age during the first months of life (53–56). It is defined by a combination of physiological recordings, electroencephalogram, electrooculogram, electromyogram, cardiorespiratory pattern, and motor activity. Several striking and precise changes, with specific timing for each, occur during this period, reflecting CNS maturation and adaptation to the extrauterine environment. Typically, the full-term newborn has significantly less indeterminate sleep, with well-differentiated active sleep (AS) and quiet sleep (QS), than do preterm infants whose indeterminate sleep may represent up to 30% of total sleep time. The architecture of the sleep–wake cycle in infants differs markedly from that of older age groups. Day–night differences in sleep–wake cycles, with an increase in the ability to remain asleep during the night and remain awake during the day for progressively longer periods, begin to arise, reflecting emergence of distinctive adult sleeping traits. These trends are relevant developmental indices of brain activity and CNS functional integrity. Information on the developmental pattern for these changes is limited by the scant data available

between 6 and 24 mon of age and by the short duration (<12 h) of recording periods in most published studies.

Following the initial observations of Fagioli *et al.* (37) on the effect of EFA deficiency on sleep–wake organization, we have included polygraphic evaluation of sleep organization during early development. Our initial findings (Uauy, R., and Peirano, P., unpublished observations) confirm previous reports (57) of less AS observed in HM-fed infants; in addition, these infants showed longer duration of QS episodes, indicating a more mature pattern of CNS development. In addition, within the randomized formula groups, we find differences in AS and QS duration and in the percentage of sleep cycles within the sleep episode.

Changes during development in spontaneous heart rate variability (HRV) patterns have been associated with sympathetic and parasympathetic interactions and utilized as an index for normal brain maturation. In healthy neonates, the variability of the high-frequency component is higher in QS than in AS, while the low-frequency component is higher in AS. During development, there is a progressive increase in HRV both during AS and QS, with specific trends for each type of HRV. There is a progressive dominance of the sympathetic tone in AS, also called rapid eye movement (REM) sleep, and a predominance of the parasympathetic vagal tone in QS or non-REM sleep. Our preliminary findings (Uauy, R., and Peirano, P., unpublished observations) support published reports of a higher HRV during AS and QS in HM-fed infants, indicating a more mature pattern of CNS development (58, 59). The effect is observed in all frequency components but is more marked in the high-frequency component, suggesting a higher parasympathetic vagal tone in the HM-fed group. Within the randomized formula groups, we find differences in HRV. These differences are not observed during wake periods.

Measurement of spontaneous motor activity using piezoelectric transducers permits continuous recording of movements during sleep and wakefulness. Observations in healthy infants demonstrate a progressive decrease in motor activity with advancing age in both AS and QS (60). We have preliminary evidence (Uauy, R., and Peirano, P., unpublished observations) indicating that HM-fed infants tend to have more spontaneous activity during sleep and that the duration of movements is longer, particularly during the early wake period.

Studies in term infants fed EFA-controlled diets. We conducted a 3-yr follow-up of healthy, full-term infants to test if HM feeding, which contains significant amounts of DHA, had an effect on the development of visual function (42). The cohorts were breast fed from birth to at least 4 mon or fed for 12 mon formula containing ample LA and 0.5% of total fat as LNA. The breast-fed group was weaned to an oleic acid (18:1)-predominant formula and received egg yolk through 12 mon of age. The breast-fed group maintained higher plasma and RBC membrane phospholipid DHA concentrations throughout the first year of life. At 3 yr of age, stereo acuity, as measured by operant preferential looking techniques, was more mature in breast-fed infants relative to the formula-fed group: 92% of the breast-fed group had mature operant preferential looking stereo acuity, whereas 35% of infants in the formula-fed group met the maturity criteria. Visual recognition in the breast-fed group was also better: while only 61% of the formula-fed infants had perfect scores, 93% of the breast-fed group had perfect scores (42).

These and other data produced by Makrides *et al.* (61) are suggestive of a DHA effect on visual maturation in term infants. Definitive proof, however, must await the final results of randomized, blind, controlled clinical trials of DHA and DHA + AA supplementation in term infants. In addition, the possibility of optimizing LNA to DHA conversion by providing sufficient LNA (>0.7% of total energy) and a lower LA to LNA ratio in formula (<10:1) may also provide for sufficient DHA for normal visual function. Initial results of Ponder *et al.* (62) indicate that this alternate strategy may be simpler and less expensive than addition of n-3 LCPUFA.

A recent study by Innis *et al.* (63) concluded that the need for DHA supplementation should not be based on the alleged improved visual acuity of breast-fed infants, since, despite differences in DHA blood contents, their study revealed no differences in preferential looking acuity between breast-fed and unsupplemented formula-fed infants. The formula used in this study contained 2.1% of total fat as LNA but no LCPUFA. However, this study used a Teller acuity card protocol for acuity assessment, a method which is less sensitive than electrophysiologic techniques to subtle visual deficits.

Other controlled studies without special reference to EFA. The first studies proposing that human breast milk feeding might exert a beneficial effect on infant psychomotor development were published during the 1920s. Since then, several studies reported small but significant advantages in developmental indices for breast-fed infants. Although no study categorically demonstrated the cause for this effect, most authors suggested that nutritional factors in human milk may account for this. Over the past decade, a renewed interest in this question has arisen, and several authors suggested that EFA may play a role in the improved developmental scores during infancy and preschool years observed in HM-fed infants. These studies were reviewed recently, but most are retrospective in nature (64).

In the Rodgers study (65), one of the few long-term follow-up studies of mental development comparing breast-fed vs. formula-fed children, where family, social, and economic variables were controlled, it was found that early breast feeding was associated with better picture intelligence at 8 yr of age and better scores in mathematics, nonverbal ability, and sentence completion at 15 yr of age. The study cohorts were selected from all births occurring during one week in 1946 in Great Britain. Based on confirmed records of early diet, 1, 133 individuals were considered entirely bottle fed, and 1, 291 individuals were never bottle fed. Functional benefits were proportional to the duration of breast feeding and remained statistically significant after controlling for social, cultural, and demographic variables by multivariate analysis. These observations could not be extrapolated to current formula-fed infants because, during the observation period, children were fed unmodified, diluted cow milk with the sole addition of sugar. Protein may have been excessive, and EFA

may have been inadequate. In a recent study by Rogan *et al.* (66), 855 newborns receiving modern formula were enrolled from 1978–1982 and followed through school age. Cognitive development was assessed with appropriate methodology and confirmed the results of the earlier British study. Breast-fed infants had significantly higher scores in the Bayley scale of mental development at 2 yr, and in the McCarthy scale at 3 and 4 yr. In addition, slightly higher English grades on report cards were found in the breast-fed group after adjusting for relevant confounding variables. A recent publication from a study in Spain confirms the benefits in mental development and the lack of an effect on motor abilities (67).

A possible long-term effect of early HM feeding on brain development is also suggested by results from a controlled trial of preterm infants indicating that HM feeding by nasogastric tube for 28 d was associated with a +8.3 point intelligence quotient difference at 8 yr of age relative to a formula-fed group after controlling for socioeconomic and other maternal variables (68). These controlled observations in preterm infants indicated that HM feeding may, in fact, offer unique advantages for brain development. More recently, a study of 526 full-term infants born in Groningen, The Netherlands, between 1975 and 1979 who were followed for 9 yr demonstrated a significantly lower occurrence of neurological dysfunction in the 135 breast-fed infants (breast fed for more than 3 wk) after adjusting for obstetric, perinatal, neonatal neurological, and social differences (69).

Differences in the early functional organization of the CNS between breast- and bottle-fed infants have also been reported. Nocturnal sleep–wake state organization at 4 mo of age show that breast-fed infants spend a higher percentage of time in QS and a lower percentage of time in AS as compared to formula-fed infants (57). Furthermore, heart rate values were lower in all sleep–wake states in breast-fed infants (70). Even though total energy expenditure during AS and QS did not differ between feeding groups, the different organization of sleep states throughout the night might account for differences in energy expenditure between feeding groups (57).

There are indications that breast-fed neonates even at the age of 2 d postpartum are better organized, physiologically, than are bottle-fed neonates (58,59). They exhibit a more organized HRV pattern and a slower heart rate in both AS and QS. They also present higher incidence of low-frequency, slower heart rate, greater HRV, and elevated vagal tone, indicating more mature HRV patterns. Conversely, breast-fed neonates were also reported to be behaviorally more irritable, more difficult to test, and less able to be consoled once crying (59).

Evidence from patients with inborn errors of metabolism. There is yet no conclusive proof for an inherited metabolic defect of DHA and or AA synthesis causing altered brain or retinal function. There is increasing evidence, however, that peroxisomal diseases may be associated with defective formation of C_{22} LCPUFA. Recently, Bennett *et al.* (71) reported fascinating but uncontrolled observations on the improve-

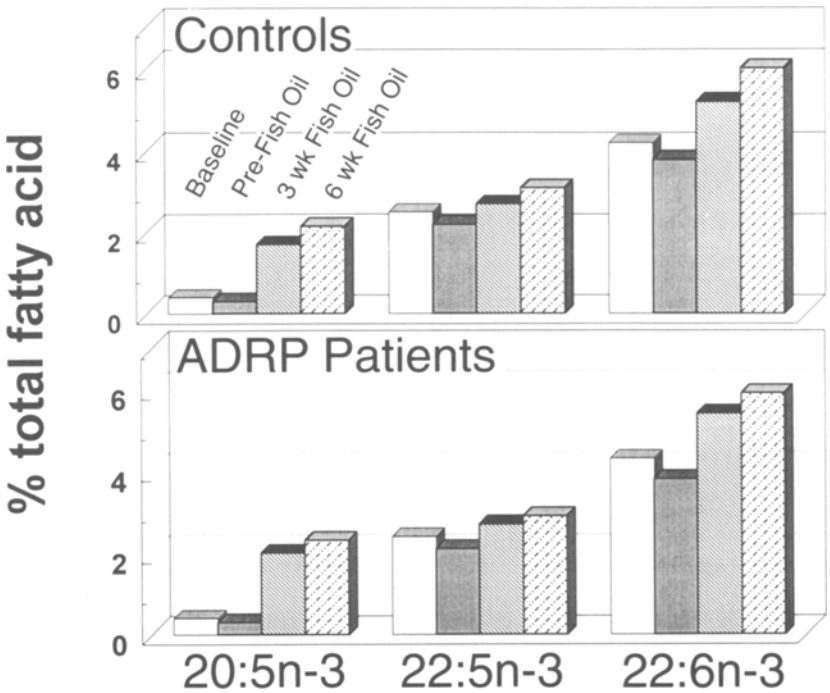


FIG. 3. Uptake of n-3 LCPUFA into red blood cell (RBC) lipids following fish oil supplementation. Levels of 20:5n-3 (EPA), 22:5n-3 (DPA), and 22:6n-3 (DHA) are reported as percent total RBC fatty acids for control and autosomal dominant retinitis pigmentosa (ADRP) subjects. Samples were obtained at baseline, after 6 wk on a controlled low-n-3 diet (pre–fish oil) and after continuing for 3 and 6 wk on the controlled diet plus daily supplementation of 3 g of a standard fish oil concentrate.

ment in neurological, retinal, and cortical electrophysiologic responses and in developmental tests in Batten's disease patients after n-3 supplementation. We studied lipid abnormalities in patients with retinitis pigmentosa (RP) and have evidence suggesting that some autosomal dominant RP (ADRP) and X-linked RP (XLRP) patients may have defective formation or incorporation of LCPUFA into membranes (72–74).

Zellweger disease. Martinez (75) provided data from several patients, indicating that, in peroxisomal disorders, brain DHA content is extremely low, plasma and RBC DHA levels are below normal, and biochemical response of these patients to fish oil supplementation is only partial. Sprecher (76) indicated that the peroxisomal β oxidation step necessary for C_{22} PUFA biosynthesis is restricted in Zellweger disease patients, since they cannot convert 24:6n-3 to 22:6n-3. Preliminary results on a limited number of patients indicate that supplementation with pure DHA is capable of preventing and also of reverting abnormal motor and mental functions associated with these conditions (77). Concomitant evidence from fibroblast studies of these patients provides supportive evidence as to the relative importance of peroxisomal β oxidation in determining biochemical and functional consequences of this disease (77).

Batten's disease. Recently, Bennett *et al.* (71) described alterations in LCPUFA metabolism in juvenile Neuronal Ceroid-lipofucsinosis, also known as Batten's disease. These patients present seizures, altered psychomotor development, retinal degeneration of rods and cones leading to blindness, motor disturbances progressing to spastic tetraplegia, and death during the first decades of life. The disease has a variable rate of progression, but in all cases it leads to mental retardation and severe motor impairment. RBC n-3 LCPUFA were found to be lower in patients with the disease relative to controls, and a significant rise was noted in these FA after marine oil supplementation (71,78). Follow-up of 6 patients after 5 to 7 yr of variable supplementation regimens demonstrated a possible amelioration in the rate of progression compared to what was predictable based on the natural history of the disease (71). Given the uncontrolled nature of these observations and the variable natural course of progression in this condition, caution should be exercised in interpreting these findings. A controlled multicenter trial is needed to clarify the clinical response to LCPUFA supplementation in these patients.

Retinitis pigmentosa. We and others have observed abnormalities in DHA metabolism in patients with RP (72–74, 79–83). RP and associated retinal degenerations are characterized by progressive night blindness and visual field constriction resulting from photoreceptor degeneration. Tissue levels of DHA and other LCPUFA may be involved in the pathophysiology of RP. There is an accumulating body of evidence that selective reductions in the plasma lipid levels of DHA may be associated with various forms of hereditary retinal degeneration (79–83). However, there were lower 18:4n-3 and higher 22:6n-3 levels in the plasma lipids of ADRP patients.

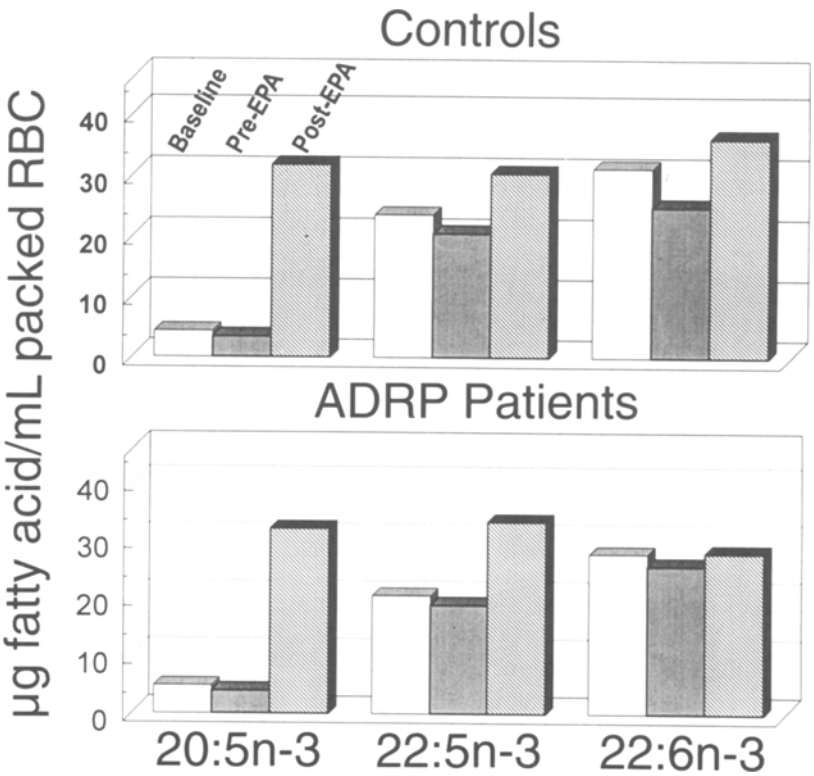


FIG. 4. Incorporation of EPA and metabolites into red blood cell (RBC) lipids following nearly pure 3 g per day EPA supplementation. Levels of 20:5n-3 (EPA), 22:5n-3 (DPA), and 22:6n-3 (DHA) are reported as μg per mL of packed RBC for control and ADRP subjects. Samples were obtained at baseline (base), after 6 wk on a controlled, low-n-3 diet (pre-EPA) and after 3 wk of the controlled diet plus daily supplementation of 3 g of EPA ethylester (post-EPA).

Segregation of patients into subgroups based on genetic inheritance type revealed that the greatest deviation from normal in plasma DHA levels was found in X-linked patients, whereas those with autosomal dominant inheritance were not significantly different from controls (84). Furthermore, specific families affected with ADRP demonstrate FA abnormalities, while other ADRP families have near normal FA profiles (80). Our results extend these findings by showing relatively similar levels of DHA in RBC among members within a family with ADRP. In ADRP, deficits in DHA levels have most frequently been identified in plasma lipids, which are rapidly modified by dietary fat intake. Abnormal lipid metabolism may result in a subnormal availability of DHA for distribution to peripheral target tissues. A limited supply of DHA for photoreceptor disc membrane structure may contribute to abnormal retinal function.

We have assessed the FA status in plasma and RBC of 50 subjects with ADRP as compared to that of 20 normal-sighted controls (72). In plasma lipids, patients with ADRP had significantly lower levels of total saturates than did the controls; derivatives of the n-6 series were significantly higher in patients with ADRP, in contrast to the lack of significant differences in n-3 FA between patients and controls. There were lower 18:4n-3 and higher 22:6n-3 in the plasma lipids of ADRP patients, however. Furthermore, a higher unsaturation index was found in the plasma lipids of patients with ADRP. In contrast to plasma, the RBC lipid FA profile of patients with ADRP compared to controls had significantly higher levels of monounsaturates and 18-carbon EFA of both the n-6 and n-3 series. Levels of n-6 LCPUFA levels were reduced in patients, particularly AA and 22:4n-6, which were markedly reduced. Overall, total n-3 LCPUFA were significantly lower than in controls. Consistent with this, the unsaturation index in RBC of patients with ADRP was reduced significantly. Full-field ERG were obtained from all patients under 50 yr of age to evaluate retinal function. Patients with lower RBC DHA levels (<3% of total FA) had significantly lower rod/cone amplitude ratios than did patients with higher RBC DHA levels (>4%) (72). Although FA changes found in ADRP patients were of moderate magnitude (<25%) compared to controls, these changes may have a significant impact on visual function if persistent over many years. This may be of particular importance for a patient who has visual function already compromised by a gene mutation.

Based on these observations, we undertook supplementation trials in a small group of patients with ADRP under controlled dietary conditions. The first trial assessed the capacity to absorb and incorporate n-3 LCPUFA as provided by a standardized fish oil concentrate. Results of this study revealed that patients did as well as controls in incorporating n-3 LCPUFA into RBC membranes, as shown in Figure 3. The second trial addressed the conversion of EPA to DHA, after oral administration of nearly pure EPA ethyl ester for 3 wk. Results of this study, as shown in Figure 4, demonstrated similar incorporation of 20:5n-3 and conversion to 22:5n-3 RBC for ADRP and control subjects after EPA supplementation. DHA in ADRP patients remained unchanged while controls exhibited a 27% higher incorporation into RBC after EPA was administered (73). These results suggest the presence of a metabolic defect in the final elongation/desaturation or β oxidation steps necessary for DHA formation from EPA.

We propose that low plasma and RBC DHA levels attributable to impaired transport or metabolism of LCPUFA may coexist with certain gene mutations and contribute to the severity of visual impairment in those with ADRP.

Our studies provide clear evidence that dietary n-3 FA deficiency affects eye and brain functions of preterm infants as measured by ERG, cortical VEP, and behavioral testing of visual acuity. Preterm infants require DHA in their diet because they are unable to form them in sufficient quantity from LNA provided by soy oil–based formula products. Dietary n-3 and n-6 FA intake resulted in discernible differences in the FA composition of plasma and RBC membrane lipids. Changes in membrane chemical structure are likely responsible for the observed functional effects. Definitive recommendations for term infants await results of randomized trials of DHA supplementation.

Preliminary evidence indicates that peroxisomal disorders, autosomal dominant and X-linked forms of RP, and possibly Batten's disease are associated with altered n-3 metabolism and may represent defects in DHA biosynthesis or incorporation into membranes. Clinical trials supplementing with pure DHA are required to evaluate this possibility further.

Our results indicate that n-3 FA are required for optimal nervous system development and for maturation of the retina in human infants and thus they should be considered essential nutrients. DHA is required by preterm infants and possibly by individuals with genetic defects in LCPUFA metabolism, such as those with peroxisomal disorders and some forms of RP.

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Effect of Dietary Docosahexaenoic Acid on Brain Composition and Neural Function in Term Infants

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ABSTRACT: There is a need to determine whether there is a dietary requirement for docosahexaenoic acid (DHA, 22:6n-3) by term infants to achieve their full developmental potential. Studies of brain fatty acid composition demonstrated that infants who were breast fed had greater levels of cerebral cortex DHA than did infants who were formula fed, suggesting that DHA in the cerebrum is dependent on a supply in the diet. Some physiological studies reported that electrophysiological and behavioral assessments of visual function were improved in breast-fed infants relative to those fed formula and that this was related to the length of breast feeding. While some randomized studies of DHA supplementation of infant formula to term infants demonstrated that the visual function of formula-fed infants could be improved to breast-fed levels by adding DHA to formula, others failed to demonstrate an effect. Variations in dietary treatments and methods of assessment make comparison of the studies difficult. Further work is necessary to rigorously establish if there are long-term benefits of dietary DHA to the term infant.
Lipids 31, 1–XXX (1996).

Breast milk is the perfect food for the growing infant, with all nutrients in an ideal balance (1–5). Despite recommendations from health professionals and learned committees (6,7), only about 75% of Australian mothers are discharged from birth centers breastfeeding their infants. Difficulties in maintaining lactation in the home results in a rapid decline in breast-feeding rates, so that, by six months, less than 30% of those who start out doing so successfully maintain lactation (8).

Infant formula is the only recommended alternative to breast milk. Since breast milk is a living tissue containing, in addition to basic nutrients, a range of cells, and important immunoglobulins, it is not possible to exactly duplicate breast milk and provide it in an easily stored form (dry powder) that can be reconstituted at will. Approximations have been made by manufacturers that reflect the reality of stability, bioavailability, and cost. Examples of approximations made by manufacturers include proteins, mineral iron, and fats. The composition of constituent amino acids of skim-milk protein varies significantly from human milk, necessitating cow milk proteins being added at a higher level (≈1.5%) than the level found in human milk (≈1%). Iron is present in very low levels in human milk (≈1 mg/L) but is extremely well absorbed (>50%). The only forms of iron that are stable in dry-powder form tend to be poorly absorbed (<10%), necessitating iron being added at many times (>10 mg/L) the level found in human milk. Fats of human milk reflect those found in human plasma and provide a complex mixture of fatty acids, including the essential 18-carbon polyunsaturates and many long-chain (20- and 22-carbon) polyunsaturated fatty acids (LCPUFA) that are proving to have important biological activity. In contrast, infant formulas contain chiefly vegetable oils containing only 18-carbon essential fatty acids, α-linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6).

Despite compromises made by manufacturers, infant formulas provide the growing infant with a basic level of nutrition that results in growth rates that are as good as or better than those observed in breast-fed infants. It has only been since neurophysiological end-points have been used that the possibility of deficiencies/limitations in the fat supply of formula-fed infants has been raised. In the past, dietary fat was considered mainly a source of energy required by the baby to support rapid growth. It is now realized that the quality and composition of fat supplied to the infant has important effects on developing organs, especially the brain. Of particular interest are the results of research on the roles of two LCPUFA, docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6).

Studies comparing formula- and breast-feeding indicated that formula-fed infants had less DHA and less AA in their erythrocytes relative to breast-fed infants. This was shown to be due in part to an imbalance in the ratio of ALA and LA. However, studies that tried to increase the availability of ALA to formula-fed infants demonstrated that it was not possible to match erythrocyte DHA levels to those of breast-fed infants (2,9). These results suggest that adding LCPUFA directly to infant formula may be necessary to match fatty acid profiles of breast-fed infants.

The relationship between brain fatty acids and diet in human infancy has also been studied. Breast-fed infants (receiving

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; LCPUFA, long-chain polyunsaturated fatty acids; PCA, post-conceptional age; VEP, visually evoked potential.

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DHA and AA) have a greater proportion of DHA in their brain cortex relative to those fed formula, largely an effect of length of feeding (10). In contrast, there are no differences in levels of brain AA between breast- and formula-fed infants. This suggests that either AA is aggressively conserved in brain tissue or that the amount of AA produced from the precursor (LA) in formula is sufficient to meet the AA requirements of the rapidly growing brain.

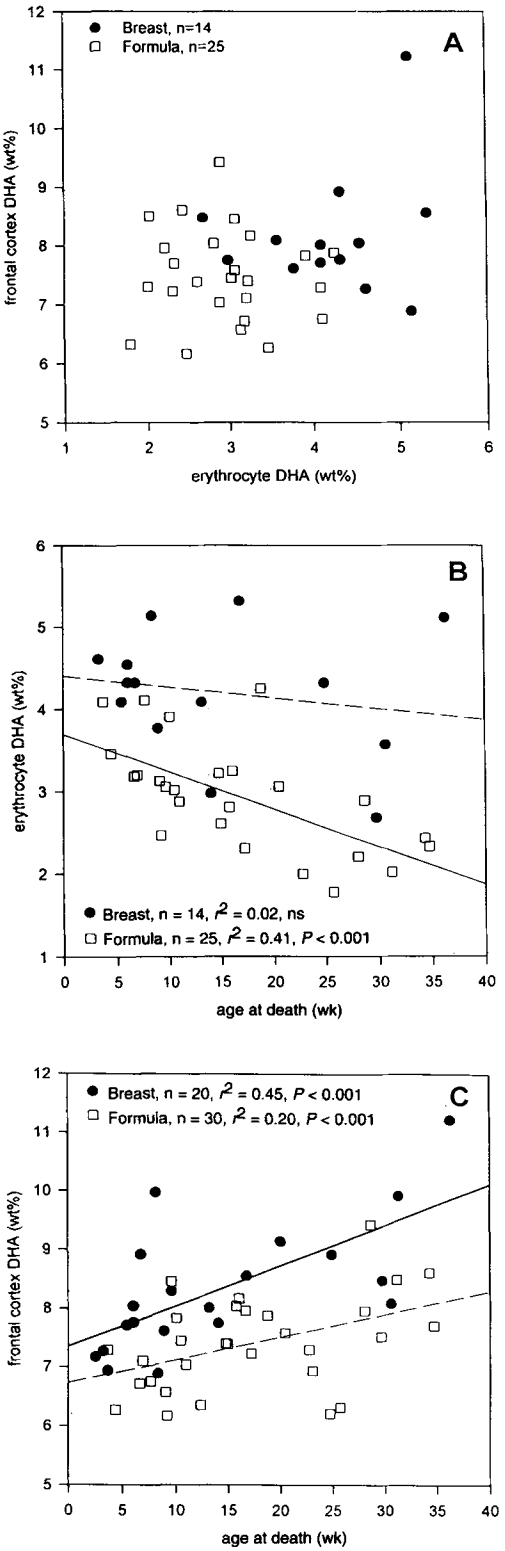


FIG. 1. A: Frontal cortex docosahexaenoic acid (DHA) as a function of erythrocyte DHA in term infants dying suddenly; B: relationship between erythrocyte DHA and age at death for breast- and formula-fed infants; C: relationship between frontal cortex DHA and age at death in breast- and formula-fed infants.

Clinical studies that associate dietary DHA with neural performance often use erythrocyte DHA as an inferred marker of brain DHA (11–13). Our results (Fig. 1) illustrate the fact that there is no simple association between cerebral DHA and erythrocyte DHA, and this may in part be explained by the different relationships among cerebral DHA, erythrocyte DHA, and age at death for breast- and formula-fed infants. For example, the proportion of cortex DHA increases with age at a greater rate for breast-fed than for formula-fed infants, while erythrocyte DHA decreases with age for formula-fed but not for breast-fed infants (Fig. 1). Despite these complicated associations, 44% of the variability in cerebral DHA could be predicted by knowing only the age at death and the level of erythrocyte DHA (10). Because both cerebral and erythrocyte DHA were influenced by diet, erythrocyte DHA may only be a valid marker of cerebral DHA if a single age is considered and if diet prior to sampling has been constant.

We examined the visually evoked potentials (VEP) of healthy term infants fed either breast milk or formula. Formula-fed infants had low erythrocyte DHA levels and reduced visual acuity, while breast-fed infants had high DHA levels and better acuity (14). We recently investigated whether differences between breast- and formula-fed term infants could be corrected by the addition of fish oil (a source of DHA) to infant formula (15). In a study involving over 70 term infants, VEP acuity in breast-fed and fish-oil-supplemented formula infants was better than in standard formula-fed infants at both 16 and 30 wk of age. Erythrocyte DHA in breast-fed and fish-oil-supplemented infants was maintained near birth levels for the entire 30-wk study period, but was reduced in infants receiving standard formula. Furthermore, VEP acuity was better in infants fed the supplemented formula or breast milk. These results are supported by clinical studies in preterm babies (12,13,16).

Most importantly, our data establish that VEP acuity is related to the length of breastfeeding. Infants breast fed for less than 16 wk had erythrocyte DHA levels intermediate between those fed breast milk continuously and those fed formula from birth when measured at 16 wk of age (Fig. 2). Furthermore, when infants were measured at 30 wk of age, only those who had received DHA for at least 16 wk had VEP acuity scores comparable to those breast fed for the entire 30 wk (Fig. 3). VEP acuity scores of those breast fed for less than 16 wk were not different than those formula fed from birth (Fig. 3). Taken together, the data indicate a need for a continuous supply of LCPUFA from breast milk for at least the first four months of life.

Growth of all infants was similar (15). AA in fish-oil-supplemented infants was reduced below levels of those fed standard formula, and this was probably exacerbated by the high levels of other fatty acids [such as eicosapentaenoic acid

(EPA), 20:5n-3] found in fish oil. This may be of some concern, as studies involving preterm infants suggest that AA is important to some aspects of growth and development (17,18).

Our results suggest that DHA levels and one visual aspect of neural performance of formula-fed infants can be normalized to breast-fed levels following supplementation with fish oil. Suppression of erythrocyte AA levels associated with the use of high-EPA fish oil as the n-3 LCPUFA supplement, however, is undesirable. Thus there is a need for a blend of fatty acids in a similar balance to breast milk, which can be safely added to formulas.

Although our results show an effect of diet on the development of the visual pathway in a group of South Australian term infants (15), other studies have not demonstrated comparable results. For example, a recent study reported by Auestad and co-workers (19) failed to demonstrate any relationship between dietary n-3 LCPUFA and visual acuity measured by sweep VEP and Teller acuity cards. These data support the study of Innis *et al.* (20), who showed that breast- and formula-fed infants have similar preferential-looking acuity development during the first three months of life. Interestingly, Carlson *et al.* (21), using formulas similar to those of Auestad *et al.* (19), demonstrated a beneficial effect of DHA on Teller acuity at two months postnatal age but not at later ages. This highlights the complexity of this area of endeavor. Uniform standards are required in study design, dietary treatments, and assessments if we are to determine whether real effects of dietary LCPUFA exist.

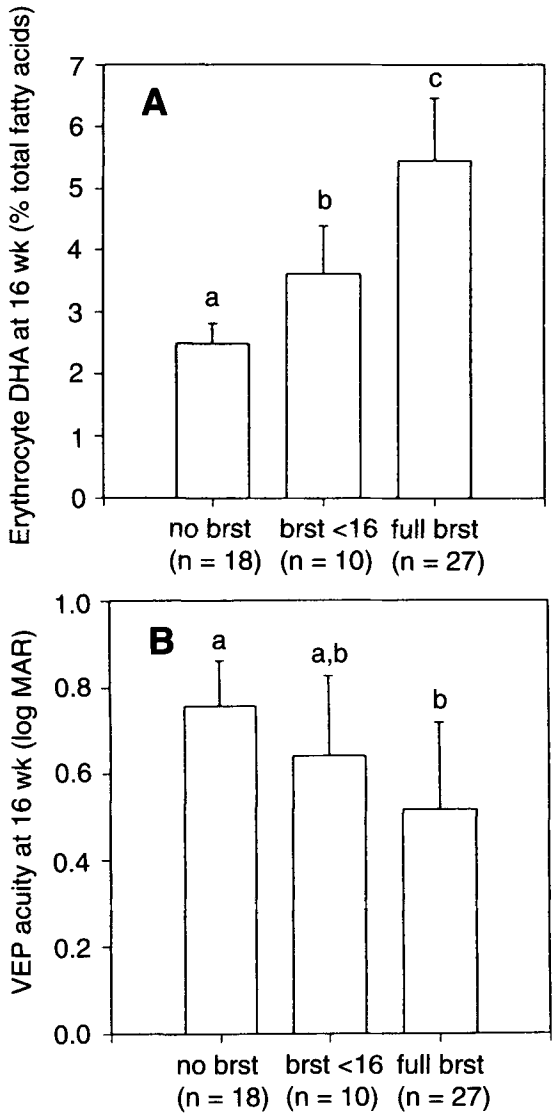


FIG. 2. Effect of breastfeeding duration on erythrocyte A: DHA ± SD and B: visually evoked potential acuity ± SD at 16 wk of age. For erythrocyte DHA, values without a common superscript a significantly different at $P < 0.0001$. VEP acuity is expressed as logMAR (log of the minimum angle of resolution), and values without common superscripts indicate significant differences at $P < 0.0005$. Abbreviation as in Figure 1.

Dietary treatments have varied widely between studies (12,13,15,16,19,21). Study formulas proposed to improve physiological outcome have contained variable DHA levels, in the presence or absence of other fatty acids, such as EPA, AA, and γ-linolenic acid (18:3n-6). The molecular type of the lipid source and the background fat blend, regarding the levels of LA and ALA, have also differed. All may affect the LCPUFA status of the infant.

Furthermore, many of these studies (12,13,16) either had no breast-fed reference groups or had breast-fed groups that received some formula. For example, Uauy *et al.* (22) complemented their human-milk reference group with increasing amounts of the fish-oil test formula, so that these infants received 50% of their energy from the test formula at four months postterm. In addition, differences exist in the DHA status of breast-fed infants (23,24), as breast milk DHA varies according to the diet of the mother.

Many of the studies have based their physiological assessments on various aspects of visual function. The electroretinogram is the specific electrical response of the retina to light and provides an assessment of the functional state of the retinal layers. Birch and coworkers (16) demonstrated an effect of n-3 LCPUFA in preterm infants at 36 wk post-conceptional age (PCA) but not at 57 wk PCA. These results concur with the findings that the retinal DHA accretion is complete by 40 wk gestation (25) and that there are no differences in retinal DHA between breast- and formula-fed infants postterm (10).

Other visual assessments, such as visual acuity, are more difficult to interpret than the results of the electroretinogram. There are both electrophysiological and behavioral ways to measure visual acuity, and acuity values obtained from such tests may be measuring different phenomena. For example, the VEP is the visually evoked response of the electroencephalogram recorded at the occipital lobe and is evoked by a moving pattern. There are several methods of VEP recording, and these largely depend on the nature of the pattern stimulator. Different patterns and different reversal rates elicit different brain responses. Studies that demonstrated differences in VEP acuity with n-3 LCPUFA status extrapolated acuity threshold from either transient or steady-state checkerboard patterns (12,15). Auestad *et al.* (19), who showed no effect of VEP acuity development with diet, assessed VEP acuity using the sweep technique with bar gratings.

Behavioral measures of acuity can be rapid and easy to use but are also subject to procedural differences. Most studies that attempted to find a relationship with n-3 LCPUFA and function used some measure of behavioral acuity. Birch *et al.* (12), using the more formal forced choice preferential looking methodology, demonstrated differences between dietary group 57 weeks PCA. Similarly, Carlson *et al.* (13,21), using a rapid acuity card technique (Teller), also demonstrated differences between treatment groups of both preterm and term infants. In contrast, other studies in term infants have failed to confirm these results using Teller acuity cards (19).

It is probably important to consider factors other than dietary DHA that may affect the DHA status of the infant including gestational age, parity, and maternal diet. The number of infants involved in the trial affect the power of the studies. For example, interpretation of preterm studies is made difficult, as there have only supposedly ever been 10 breast-fed preterm infants (22). It is difficult to extrapolate studies on visual function to developmental milestones. Developmental assessments such as Bayley's scales are strongly influenced by socioeconomic class, maternal education, and amount of stimulation the child receives in the home; these must all be controlled before the effect of any dietary treatment can be tested. Overcoming the confounding influences will require well-controlled studies involving large numbers.

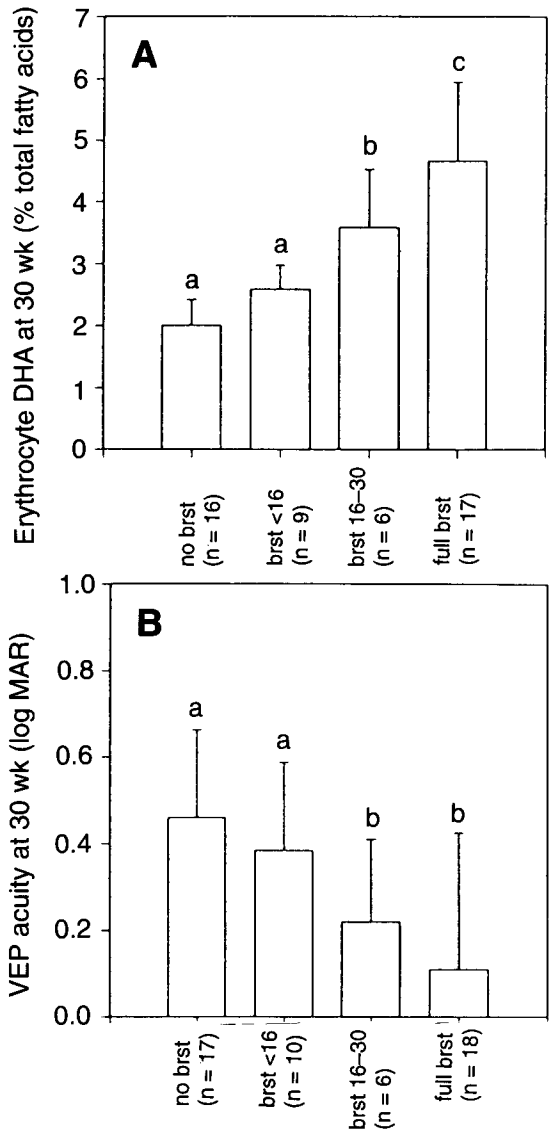


FIG. 3. Effect of breastfeeding duration on erythrocyte A: DHA ± SD and B: VEP acuity ± SD at 30 wk of age. For erythrocyte DHA, values without a common superscript are significantly different at $P < 0.0001$. VEP acuity is expressed as logMAR (log of the minimum angle of resolution), and values without common superscripts indicate significant differences at $P < 0.001$. Abbreviations as in Figures 1 and 2.

There are some tantalizing clues that suggest that early nutrition is related to long-term outcome. Barker *et al.* (26,27) reported that nutritional stress before the baby is born can be a determinant in adult cardiovascular disease, and the work of Lucas *et al.* (28) suggests that breast-milk consumption by preterm infants for the first four weeks of life is related to improvements in intelligence quotient at 7–8 y of age. In the absence of additional information, it may be circumspect to assume that a supply of LCPUFA is important, since LCPUFA accumulate in the brain when provided and are present in breast milk.

Formula manufacturers have released formulas containing LCPUFA, generally in the form of a phospholipid fraction isolated from egg yolk. They are apparently stable to oxidation in powder form but increase levels of phosphorus and choline (from the phospholipid) in the formula. Experimental batches of formulas with fish oils as sources of LCPUFA proved to be unstable in dry-powder form; however, the AA and DHA of breast-milk fats are in the form of triglycerides. Addition of LCPUFA to preterm formulas has been recommended by authorities in Europe (7) and Canada (6). The question to be addressed is whether there is a sufficient body of evidence supporting inclusion of LCPUFA in the diets of infants.

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Increased Docosahexaenoic Acid Levels in Human Newborn Infants by Administration of Sardines and Fish Oil During Pregnancy

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ABSTRACT: In rhesus monkeys, maternal n-3 fatty acid deficiency during pregnancy produces infant monkeys deficient in n-3 fatty acids at birth. These results stimulated current experiments to find out if n-3 fatty acids from fish in the diets of pregnant women would influence the concentration of docosahexaenoic acid (DHA, 22:6 n-3) in the newborn human infant.

Fifteen healthy pregnant women were enrolled to receive a 9-wk dietary supplementation of n-3 fatty acids from the 26th to the 35th wk of pregnancy. Sixteen pregnant women were not supplemented and served as controls. n-3 Fatty acid supplementation consisted of sardines and additional fish oil, which provided a total of 2.6 g of n-3 fatty acids per day (d) for the 9-wk period of supplementation. This included 1.01 g DHA.

The end point of this study was the blood concentrations of DHA in the newborn infant. DHA in maternal red blood cells increased from 4.69% of total fatty acids to 7.15% at the end of the supplement period and at the time of delivery decreased (as expected) to 5.97% of total fatty acids. Maternal plasma showed a similar change from 2.12 to 3.51% of total fatty acids and then decreased to 2.35%. Levels of DHA in plasma and red blood cells of unsupplemented mothers did not change during the same time period.

Levels of DHA in blood of newborn infants differed greatly in infants born from n-3–supplemented mothers compared with control infants. In red blood cells, DHA was 7.92% of total fatty acids compared with 5.86% (control infants). Plasma values showed a similar difference: 5.05% vs. 3.47% (controls). In n-3–supplemented infants, DHA concentrations were 35.2% higher than in control infants in red blood cells and 45.5% higher in plasma.

These data indicate the importance of maternal dietary n-3 fatty acids and, in particular, maternal dietary DHA in promoting higher concentrations of DHA in the blood of the newborn infant.
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The growth of the brain occurs in two phases: prenatal and postnatal. During intrauterine life, the fetal brain accumulates long-chain polyunsaturated fatty acids, docosahexaeonoic (DHA, 22:6 n-3) and arachidonic acid, which are vital components of phospholipid membranes of the newborn infant (1). These fatty acids are transported from maternal circulation across the placenta into fetal venous blood (2), from which they subsequently cross the blood–brain barrier into the central nervous system (3). The quantity of DHA that maternal circulation must supply to the fetus is considerable. The fetal brain acquires approximately 21 g/wk during the last trimester of pregnancy (4). Even more DHA, however, is stored in the white adipose tissue, 326 mg/wk.

In monkeys fed a diet deficient in n-3 fatty acids, the amount of DHA found in the maternal circulation is much lower than normal. Thus, newborn monkeys born from deficient mothers have low contents of DHA in blood and tissues, including brain and retina (5). When the n-3–deficient diet was continued after birth, infant monkeys developed impaired vision and abnormal electroretinograms (6). Premature human infants also have impaired visual function when fed standard infant formulas (7,8). Vision in these premature human infants improved considerably when fish oil,

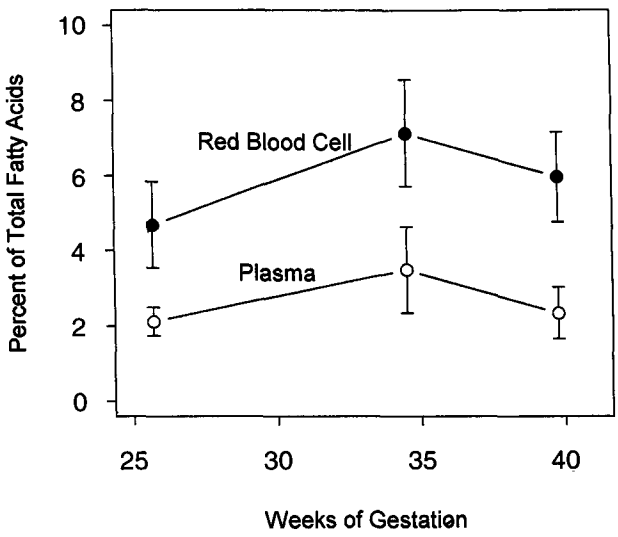


FIG. 1. Plasma and red blood cell docosahexaenoic acid (DHA) in supplemented mothers (× ± SD) at study entry (before supplementation), at end of supplementation (34 wk gestation), and at

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

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supplying DHA and other n-3 fatty acids, was incorporated in their formulas (8). The data in premature human infants are of interest in the context of the present study because at birth blood and tissues of premature infants represent earlier timepoints in the life of the full-term infant during intrauterine growth and development.

This evidence that newborn infant monkeys are n-3 fatty acid–deficient when the maternal diet is deficient stimulated development of an experiment in pregnant women to ascertain if increasing the amount of DHA in maternal diet and then in maternal circulation would influence blood levels of DHA in the newborn infant. Our hypothesis was: would higher dietary intakes of DHA in pregnant women increase the DHA content in their newborn infants? This fatty acid is of special interest because of its importance in the development of the brain and retina.

METHODS

Volunteers. Thirty-one healthy pregnant women and their newborn offspring participated in this study. All women participating in the study signed consent forms approved by the Committee on Human Research of Oregon Health Sciences University (OHSU, Portland, OR).

Supplemented group. Healthy pregnant women attending the Obstetrical Clinic of the University Hospital (Portland, OR) were invited to participate in a study that involved increasing their consumption of n-3 fatty acids in the form of sardines and fish oil. Women were recruited at approximately 24 wk gestation but were accepted into the study until 30 wk gestation. Fifteen women consented to the study, were supplemented, and successfully completed the study.

Unsupplemented (control) group. Healthy pregnant women were recruited to participate as control subjects with no supplementation. Control volunteers were seen in a separate clinic area from supplemented women.

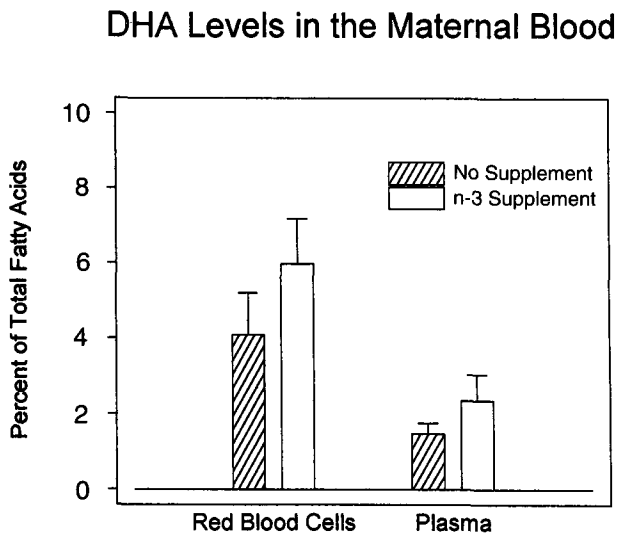


FIG. 2. Red blood cell and plasme docosahexaenoic acid (DHA) levels in supplemented and unsupplemented mothers at delivery. Red blood cell DHA was significantly different at $P < 0.0042$. Plasma DHA was significantly different at $P < 0.0042$.

TABLE 1
Fatty Acid Composition of n-3 Fatty Acid Supplements

	Percent of total fatty acids	
Supplement	Sardines	Fish oil
EPA[a]	7.5	17.9
DHA[b]	7.1	12.0
Total n-3	19.2	36.0
Total n-6	24.7	3.3
Monounsaturated	40.0	29.2
Saturated	13.6	30.4

[a]EPA = Eicosapentaenoic acid.
[b]DHA = Docosahexaenoic acid.

Supplementation. The supplementation period began at entry (24 to 30 wk gestation) and continued until 34 wk gestation at which time supplementation was discontinued. The goal of supplementation was to achieve at least 3 g/d total n-3 intake or about 1.1 g DHA. The women enrolled in the supplement group were asked to consume a combination of sardines and fish oil: $\frac{1}{2}$ can of sardines per d plus 7 fish oil capsules (1 g each) or 1 can of sardines per d plus 3 fish oil capsules. Sardines were supplied to the women in $3\frac{3}{4}$-oz tins. Those who did not tolerate sardines were asked to take 10 fish oil capsules per d in a divided dose. Women were encouraged to eat the recommended amount of sardines and supplement but were not dropped from the study if they were unable to comply fully. They were asked to report only what they did consume. Women recorded their intake of supplements and sardines on provided intake forms. These were returned at each visit. Based on the total quantity of each type of sardines eaten and number of fish oil capsules consumed, a total intake for the supplement period was calculated. Fish oil was provided by Hall Laboratories, Inc. (Portland, OR). Sardines were provided by the North Atlantic Sardine Council (Seattle, WA). Fatty acid composition of these products is listed in Table 1.

Diet assessments. The habitual dietary n-3 fatty acid intake of the pregnant women was assessed by a detailed food frequency questionnaire that identified 30 commonly eaten fish and seafood items and asked the women to identify the amounts of each item eaten in the past month.

Sample analysis. Blood samples were collected from all mothers at entry, monthly after entry, and at delivery. A sample of the infant's blood was obtained from residual blood in the umbilical cord. Samples collected at delivery were refrigerated and taken to the laboratory for analysis within 24 h. Plasma was separated immediately after centrifugation. The erythrocyte pellet was washed three times with saline. Fatty acids of lipid extracts of plasma and erythrocytes were liberated by incubation with 6% ethanolic KOH and then extracted with hexane. After methylation, fatty acids in extracts were quantitated by capillary gas–liquid chromatography as described previously (2). An internal standard of heptadecanoic acid was added to both erythrocyte and plasma extracts. Peaks were identified by computerized comparison with standards, which were run daily. Plasma and erythrocyte fatty acid concentrations are given in percent of total fatty acids.

Statistical analysis. Gestation in weeks was calculated for each study visit. Individual fatty acids for plasma and erythro-

cytes were averaged for entry, end of supplementation (34 wk gestation), and delivery. In one mother who had triplets, fatty acids of the three cord blood samples were averaged and considered as one analysis. Fatty acids for mothers in the supplemented and control groups were compared by *t* test at each time-point. Infants' fatty acids at delivery were also compared by *t* test. The Bonferonni correction for multiple *t* test comparisons was made: *P* value for fatty acid comparisons of mothers were multiplied by 42 (14 fatty acids each compared 3 times) and infants' *P* values were multiplied by 14 (14 fatty acids compared once). The level of significance was $P < 0.05$ for corrected *P* values.

RESULTS

Amounts of n-3 fatty acids consumed by both control and supplemented pregnant women. Consumption of DHA and eicosapentaenoic acid (EPA) in the usual diet of both groups of pregnant women was small and comparable in both groups: 2.5 ± 1.5 g/mo in supplemented mothers and 2.82 ± 2.2 g/mo in control mothers. Food sources of these fatty acids were largely from fish and chicken. Supplemented mothers, however, ingested 147 ± 59 g n-3 fatty acids from sardines and fish oil over the entire period. The range was 45 to 219 g. The daily amount of n-3 fatty acids was 2.6 g (range 0.95–3.1 g) and included 20:5, 22:5, 22:6n-3 fatty acids. Of the total 147 g ingested, the fatty acid breakdown between DHA and EPA was as follows: 59.4 g and 75.7 g, respectively.

Consumption of sardines depended on tolerance of the women. They were given new recipes that incorporated sardines. It was emphasized that sardines, besides being rich in n-3 fatty acids, contained ample amounts of protein and calcium. Sardine consumption was from 0–1 can per d. Fish oil capsules (1 g each) taken from 3–10 g per d depended on the quantity of sardines consumed, with more fish oil given to women who took no or very small quantities of sardines. Enrichment of n-3 fatty acids in the diet from sardines and fish oil was roughly 30-fold compared to the usual ingestion. Duration of the supplement averaged 56 d with a range of 30–81 d. No supplement of sardines or fish oil was prescribed after the 34th wk of pregnancy.

All supplemented mothers and control mothers had normal deliveries. Supplemented mothers were advised to reduce other fats in the diet because of the extra consumption of fat from fish oil and sardines.

Enrichment of DHA in plasmas and red blood cells of supplemented pregnant women. The focus of this study was on DHA, since it is the key fatty acid of the n-3 series most prominent in the brain and retina. In supplemented mothers, plasma DHA was 2.1% of total fatty acids at baseline and 3.51% at the end of supplementation some 56 d later (Fig. 1). At the time of birth, plasma values were still elevated about 11% over baseline, with DHA totaling 2.35% of total fatty acids. DHA of maternal red blood cells paralleled plasma DHA, with levels of 4.69% of total fatty acids at baseline, 7.15% at the end of supplementation, and 5.97% at the time of delivery. DHA of plasma and red blood cells of control mothers did not change from baseline to delivery. At delivery, their plasma DHA was 1.47% of total fatty acids, and red blood cell DHA was 4.08%. Thus supplemented mothers, despite taking no extra n-3 fatty acids for the last six weeks of pregnancy, still had blood levels (plasma and red blood cells) very much higher than control mothers (Fig. 2 and Table 2).

Increased DHA concentrations in blood of newborn infants from supplemented mothers. There were distinct differences in DHA concentrations in blood of infants from supplemented mothers vs. infants from control, unsupplemented mothers (Fig. 3). These differences appeared in both plasma and red blood cells. DHA content of plasma was 5.05% of

TABLE 2
Comparison of Plasma and Red Blood Cell DHA in Control, Unsupplemented, and n-3 Fatty Acid-Supplemented Mothers[a]

	Unsupplemented mothers (n = 16)	Supplemented mothers (n = 15)	*P* Values[b]
Total supplementary			
n-3 fatty acids (g)	0	147 ± 59	
DHA (g)	0	59.4	
EPA (g)	0	75.7	
Baseline values			
Gestation (wk)	28.99 ± 2.87	25.67 ± 2.72	
Plasma DHA (%)	1.63 ± 0.34	2.12 ± 0.38	0.0504
Red blood cell DHA (%)	3.74 ± 0.66	4.69 ± 1.14	0.6720
At end of supplementation			
Gestation (wk)	34.84 ± 1.88	34.50 ± 0.56	
Plasma DHA (%)	1.60 ± 0.73	3.51 ± 1.15	0.0042
Red blood cell DHA (%)	4.07 ± 0.68	7.15 ± 1.42	0.0042
At delivery			
Gestation (wk)	40.18 ± 1.29	39.76 ± 1.58	
Plasma DHA (%)	1.47 ± 0.29	2.35 ± 0.69	0.0042
Red blood cell DHA (%)	4.08 ± 1.13	5.97 ± 1.19	0.0042

[a]See Table 1 for abbreviations.
[b]*P* values adjusted to correct for multiple comparisons.

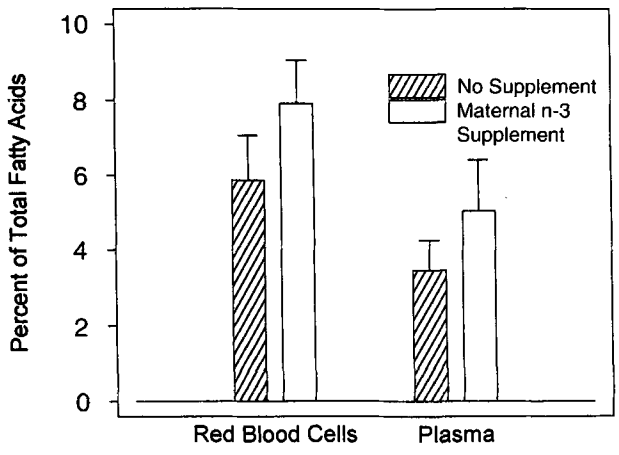


FIG. 3. Red blood cell and plasma docosahexaenoic acid (DHA) levels at delivery in newborn infants from n-3–supplemented and –unsupplemented mothers. Red blood cell DHA significantly different at $P < 0.001$. Plasma DHA was significantly different at $P < 0.011$.

total fatty acids in infants from supplemented mothers *vs.* 3.47% in infants from control mothers. "Supplemented" infants had plasma DHA levels some 45.5% higher than controls. Red blood cells of the newborn "supplemented" infants likewise showed similar increases of DHA: to 7.92% of total fatty acids vs. 5.86% of fatty acids in control infants, or 35.2% greater. These plasma and red blood cell DHA differences were highly significant.

Comparison of the mother at delivery who had taken the least amount of n-3 fatty acids with the mother who consumed the highest amount. The least amount of n-3 fatty acids consumed in a supplemented mother was only 45 g over 47 d, or an average of 0.95 g/d (Table 3). This mother can be contrasted with the mother who took the most amount of n-3 fatty acids, 219 g over a 70-day period. Her daily amount, 3.1 g, was three times greater than levels in the mother taking the least amount. Plasma DHA was 1.52% of total fatty acids (least consumption) compared to 2.39% (most). Similar comparisons for red blood cells were 5.20% (least) vs. 6.18% (most). Newborn infants from these mothers had greatly different plasma values. Plasma values were 7.59% (most) vs. 3.3% (least), more than two times the levels in infants from the "least" mothers. Newborn red blood cells showed a similar divergence: 9.91% (most) vs. 6.56% (least). In general, then, the amount of DHA in infants' plasma and red blood cells paralleled roughly the amount present in maternal blood and was related to the amount of n-3 fatty acids ingested in fish oil or sardines.

TABLE 3
Comparison of DHA in Newborn Infants and Mothers Who Had Received the Least and Most Amounts of n-3 Supplements[a]

	No supplement	Supplemented mothers	
		Least	Most
Amount of n-3 fatty acid intake (g)	0	45.0	219.0
DHA (g)	0	17.4	87.1
EPA (g)	0	23.6	114.5
Maternal			
Red blood cells (%)[b]	4.08 ± 1.13	5.20	6.18
Plasma (%)	1.47 ± 0.29	1.52	2.39
Newborn infant			
Red blood cells (%)	5.86 ± 1.20	6.56	9.91
Plasma (%)	3.47 ± 0.80	3.33	7.59

[a]See Table 1 for abbreviations.
[b]Percent of total fatty acids.

DISCUSSION

n-3 Fatty acids containing considerable quantities of DHA increased the DHA levels in both plasma and red blood cells of mothers supplemented with sardines and fish oil from midpregnancy to the 34th wk. DHA and other n-3 fatty acids were transferred across the placenta into the fetus so that at the time of birth newborn blood levels of DHA were 35% higher than levels in newborn control infants. Placental transport of DHA has been demonstrated by studies of blood taken from the umbilical cord at the time of delivery (2). There was a considerable venous–arterial difference in levels of DHA and other n-3 fatty acids. These differences were particularly noted in the red blood cells, thus raising the question about red blood cell as a prominent carrier of DHA in fetal blood.

This study was safe for both mothers and infants. Supplemented pregnant women had normal births and delivered normal full-term infants. Length of pregnancy was not affected by this kind of supplementation. The study proved the practicality of increasing levels of DHA in blood of pregnant women and then also in blood of newborn infants through dietary supplementation. Higher quantities of DHA in infants' blood would suggest higher levels also in their tissues. This greater quantity of DHA was available during the period of maximal brain and retinal development (the latter half of pregnancy). This is particularly important, since it has been shown that premature infants born during the last trimester of pregnancy have low levels of DHA in the blood and have impairment of visual development (7,8). Addition of fish oil containing DHA to formulas for premature infants increased blood DHA and improved vision (7,8). It is reasonable to hypothesize that, had the mothers of these premature infants received n-3 fatty acid supplementation during pregnancy, then their infants might well have been better off in terms of retinal and brain development at the time of their premature births.

There are several other potential benefits of n-3 fatty aid supplementation from sardines and fish oil. These are prevention of premature labor and preeclampsia. From observations in the Faroe Islands, where considerable quantities of marine oil are consumed, it has been suggested that n-3 fatty acids from marine oil might prolong gestation through inhibition of prostaglandin synthesis (10,11). This would be of value to women with habitual premature labor. Prematurity in the Faroe Islands was certainly less than in Denmark. There were more stillbirths in the Faroe Islands than in Denmark, however. Whether supplemental n-3 fatty acids would prevent premature births remains open to question. Our study, however, which was not designed to affect the onset of labor, demonstrated that supplementation with n-3 fatty acids was perfectly safe from midpregnancy to the 34th wk. There are also some indications that the incidence of preeclampsia can be reduced by fish oil consumption during pregnancy (13).

ACKNOWLEDGMENTS

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Polyunsaturation in Cell Membranes and Lipid Bilayers and Its Effects on Membrane Proteins

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ABSTRACT: The effect of variation of the degree of *cis*-unsaturation on cell membrane protein functioning was investigated using a model lipid bilayer system and protein kinase C (PKC). This protein is a key element of signal transduction. Furthermore it is representative of a class of extrinsic membrane proteins that show lipid dependent interactions with cell membranes. To test for dependence of activity on the phospholipid unsaturation, experiments were devised using a vesicle assay system consisting of phosphatidylcholine (PC) and phosphatidylserine (PS) in which the unsaturation was systematically varied. Highly purified PKCα and ε were obtained using the baculovirus-insect cell expression system. It was shown that increased PC unsaturation elevated the activity of PKCα. By contrast, increasing the unsaturation of PS *decreased* the activity of PKCα, and to a lesser extent PKCε. This result immediately rules out any single lipid bilayer physical parameter, such as lipid order, underlying the effect. It is proposed that while PC unsaturation effects are explainable on the basis of a contribution to membrane surface curvature stress, the effects of PS unsaturation may be due to specific protein-lipid interactions. Overall, the results indicate that altered phospholipid unsaturation in cell membranes that occurs in certain disease states such as chronic alcoholism, or by dietary manipulations, are likely to have profound effects on signal transduction pathways involving PKC and similar proteins. *Lipids 31,* S-189–S-192 (1996).

Over the past decade there has been intense study of the effects of altered membrane unsaturation on cell structure and function. Although much is now known about the effects of unsaturation on cell membrane physical properties [recently reviewed (1)], the manner in which membrane proteins are influenced by varying degrees of polyunsaturation is still not clear. Recently, the focus has shifted to examination of the effect of unsaturation at discrete regions of the membrane. For example, effects that are localized at the protein-lipid interface, or other regions of defined depth in the bilayer or in laterally separated regions of the membrane (see for e.g. Refs 2–4). The effects of increased unsaturation in a lipid bilayer include decreased lipid acyl chain order (5) and increased hydration in the head group and interchain regions (2,4,6–9). It has been proposed (4,8) that altered hydration may have marked effects on membrane protein functioning, possibly due to the formation of hydrogen bonds between the interchain water and protein amino acid side chains facing into the hydrophobic interior of the membrane.

Many of the key proteins involved in signal transduction including, for example, phospholipase A_2, phospholipase Cγ, G-proteins, raf-kinase, synaptotagmin and protein kinase C, are extrinsic membrane proteins. Some of these translocate from the cytoplasm to the cell membrane as part of early signaling processes that initiate a cascade of events that lead to

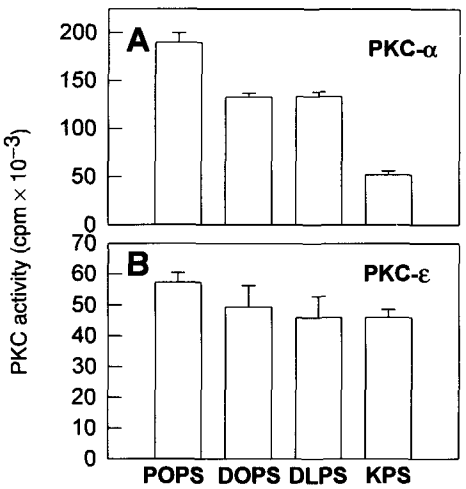


FIG. 1. Effect of phosphatidylserine (PS)-unsaturation on protein kinase C (PKC) activity. Relative activities of (a) PKCα and (b) PKCε for different PS-molecular species. The phosphorylation of MBP_{4-14} (PKCα) or PKCε-peptide using [^{32}P]ATP determined using 150 μM lipid vesicles comprised of PS/phosphatidylcholine (1:4, molar) for palmitoyl-oleoyl-PS (POPS), dioleoyl-PS (DOPS), dilinolcoyl-phosphatidylcholine (DLPS) and krill-PS (K-PS) using 0.5 μM 4B-12-O-tetradecanoyl. Experimental details are as described in the Procedures section.

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Abbreviations: B-PS, brain-PS; DLPS, dilinoleoyl-PC; DOPS, dioleoyl-PS; DPH, 1,6-diphenyl-1,3,5-hexatriene; K-PS, krill-PS; LUV, large (100 nm diameter) unilamellar vesicles; MBP_{4-14}, myelin basic protein; PC, phosphatidylcholine; PKC, protein kinase C; POPC, palmitoyl-oleoyl-PC; POPS, palmitoyl-oleoyl-PS; PS, phosphatidylserine; SUV, small (30 nm diameter) unilamellar vesicles; TPA, 4B-12-O-tetradecanoylphorbol-13-acetate.

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events such as cell proliferation, differentiation, etc. It is becoming apparent that this interaction with the membrane is not only lipid dependent but that it depends on the membrane lipid composition. PS is believed to be a prominent mediator of the interaction of these types of proteins with the cell membrane. However, while PS interacts directly with the protein, as we have shown recently (10), other cell membrane lipids, including PC and PE, may influence this process indirectly due to effects on membrane lipid bilayer structure. The compositional aspect that seems to be important here is the degree of unsaturation of the phospholipid. The role of the *type* of unsaturation (i.e., n-3 or n-6) or distribution of double bonds along the chain remain unknown. Recent studies from this laboratory have focused the effect of varied unsaturation on the activity of PKC as an important example from the above list of proteins. Studies published so far by this laboratory and others indicate that both PC (10,11) and PS (11–13) unsaturation influence PKC activity.

The first study that investigated PS unsaturation and PKC activity, with n-3 rich trout liver as compared to bovine brain PS (13), concluded that PKC activity was decreased when PS-unsaturation increased. In another study, the opposite was concluded, that increasing the PS unsaturation (mixed species from brain) increased *maximal* PKC activity. It was shown that the effect was not due to the amount of bilayer bound PKC nor due to lateral phase separation. Also electron micrographs of the vesicles (see also (14)) showed vesicle integrity was maintained. More recently, mixed PKC species from rat brain was investigated using a multilamellar vesicle system in which PS and PC unsaturation was varied (11). Increased unsaturation led to elevated PKC activity and, in this case, PS unsaturation was also found to increase activity. However, in that study the PS unsaturation did not extend to greater than one double bond/PS. Fully (di)-saturated-PS supported only low levels of activity and was obtainable only in the presence of *unsaturated* PC. Thus, if *both* PS and PC were saturated, no activity was obtained. Overall, a roughly linear correlation between the percentage of unsaturated phospholipid and PKC activity was indicated. Interestingly, membrane fluidity did not appear to play a part since cholesterol, which greatly reduces fluidity, had little effect on PKC activity and it was reasoned that the physical parameter of importance was "head group spacing". All of these studies were complicated by the use of non-optimal vesicles systems, mixed PKC isoform preparations and the aggregatory substrate histone. Therefore, at present there is no general agreement on the effects of PS-unsaturation on PKC activity.

Recently, we found that increased PC unsaturation results in a potentiation of PKC activity in defined vesicle systems (SUV or LUV). It was further proposed that the parameter, instead of solely a function of head group spacing, was also connected with the energetics of head group interactions that *include* the tendency of some lipids, notably PE along with high unsaturation, to form non-bilayer phases, and quantified as "curvature stress" (see for e.g. Refs 15–17). Thus, when PE and highly unsaturated lipids are constrained to form a bilayer, the system gains potential "curvature" energy which may be utilized to favor the interaction of the bilayer with proteins such as PKC, a process that tends to relieve the stress energy. Studies from this laboratory (10) showed that increasing PC unsaturation increased activity. When PE was included, activity at low PC unsaturation was elevated but that at high unsaturation reduced. This was rationalized from further experiments in which PE was varied in dioleoyl-PC bilayers where it was found that the activity increased to a maximum but then declined. It was concluded that PKC activity is therefore a *biphasic* function of the effect of increasing PE and unsaturation, which is to increase head group spacing and curvature stress. Again, this study used a mixed isoform preparation although the assay system was non-aggregatory. A model in which head group spacing or a related parameter is the major factor dictating PKC activity is compatible with monolayer studies (18,19) which showed PKC activity depends on the surface pressure. In the latter, a biphasic profile of activity as a function of surface pressure was found that would be compatible with our results using bilayer systems.

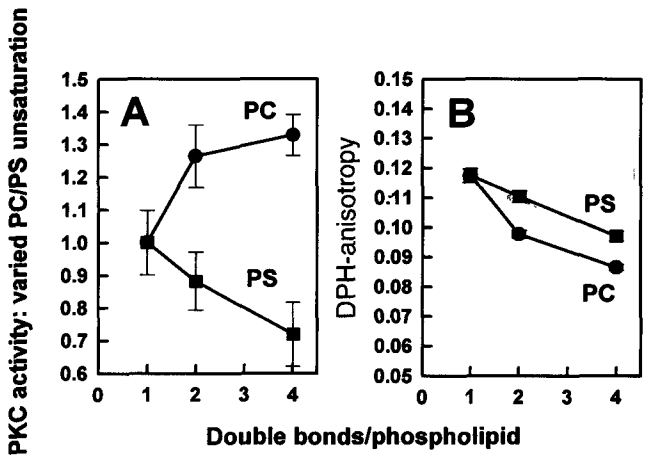


FIG. 2. Phosholipid unsaturation effects on PKC activity not explainable on the basis of lipid order. A: PKCα activity as a function of the double bonds/molecule of phosphatidylcholine (PC) or PS in PC/PS (4:1, molar) vesicles (details as described in Fig. 1). B: The fluorescence anistotropy (using DPH: 1,6-diphenyl-1,3,5-hexatriene), reflecting the lipid order, as a function of PS/PC unsaturation. Experimental details are as described in the Experimental Procedures section. Abbreviations as in Figure 1.

Considering that PS is required for PKC activation, and the fact that its fatty acid composition is highly susceptible to dietary manipulation, the finding of contradictory reports on the effects of PS unsaturation on PKC reveal the need for further study. It may be conjectured that the effects of increasing PS unsaturation might either follow that found with PC or may involve more specific protein-lipid interactions. To test this, we examined the effects of PS with varying levels of unsaturation on highly purified recombinant-PKCα isoform using a stable and non-aggregatory PKC assay system. The results indicate that increasing PS unsaturation decreases PKC activity, a finding that is opposite to the effects of PC unsaturation.

EXPERIMENTAL PROCEDURES

Materials. Myelin basic protein (MBP_{4-14}) was from LC Services (Woburn, MA). The PKCε pseudosubstrate peptide was custom synthesized by Biosynthesis, Inc. (Lewisville, TX). Lipids (from Avanti Polar Lipids, Inc., Alabaster, AL) included POPC, POPS, brain-PS (major fatty acids as mole %, 16:0 1.4; 18:0 42.2; 18:1 26.9; 18:2 4.2; 20:4 2.9; 22:6 9.7) and krill-PS (major fatty acids, 16:0 25.6, 18:0 1.3, 18:1 3.7, 18:2 1.3, 20:4 0.9, 20:5 37.9, 22:6 18.3). TPA was from Sigma (St Louis, MO). Adenosine 5' triphosphate (ATP) was from Boehringer Mannheim (Indianapolis, IN) and [γ^{32}P]ATP was from New England Nuclear (Boston, MA). All other chemicals were of analytical grade and were obtained from Fisher Scientific (Malvern, PA). DPH was from Molecular Probes (Eugene, OR).

PKC expression and purification. Recombinant PKCα (bovine) was prepared using the baculovirus-insect cell expression system as previously described (20,21). PKCε was prepared by subcloning the cDNA, obtained from a rat brain cDNA library, into a baculovirus expression vector (Yeager, M.D., Taddeo, F.J., Slater, S.J., and Stubbs, C.D., to be published). The infection of Sf9 insect cells and purification of the PKC was according to established procedures (20,21).

Preparation of vesicles. Lipids in chloroform (750 μM), in the presence of DPH (when required for lipid order measurements; DPH:phospholipid, 1:400, molar) were placed in a test tube and the solvent removed under a stream of nitrogen. The mixture was vortexed in 50 mM Tris/HCl buffer, pH 7.4 to form multilamellar lipid vesicles. Large unilamellar vesicles (LUV) were made by the extrusion technique, using an Avestin Liposofast Extruder (MM Developments, Ottawa, Canada) as previously described (22). Vesicles of a similar composition have been previously shown to be stable under the PKC-assay conditions used here (14)..

PKC activity. Activity was determined from the initial rate of incorporation of ^{32}P from [γ^{32}P]ATP (New England Nuclear) into a peptide substrate, according to a previously detailed method (10) modified from an earlier method (23). For PKCα, a peptide corresponding to the phosphorylation site of MBP_{4-14} (50 μM) was used. For PKCε activity (calcium-independent), the assay mixture contained 0.1 mM EGTA and an e-peptide in place of the MBP_{4-14}, that was based on the pseudosubstrate sequence of PKCε (24,25), a single alanine residue was replaced by a serine.

Fluorescence measurements. The steady state anisotropy of DPH was determined using an SLM 48000 fluorimeter in the T-format as previously described (2).

RESULTS AND DISCUSSION

The effect of increasing PS unsaturation in PC/PS unilamellar vesicles (4:1, molar) on recombinant-PKCα is shown in Figure 1A. It was found that increasing the level of unsaturation decreased the activity of PKC in this system. This contrasts with the results of our previous study where increasing PC unsaturation was shown to increase the activity of PKC. The PKC used in that study was a rat brain PKCα,β,γ (Ca^{2+}-dependent) mixture. However, here, the same effect was demonstrable with the recombinant-PKCα enzyme (Fig. 2A). The krill-PS, commercially obtained, is extremely rich in 20:5 and 22:6n-3 (see Methods) and gave a much reduced activity compared to the other PS species.

A major determinant of the PS and Ca^{2+} requirement in PKC is contained within the C2-domain of the enzyme. This region contains the main Ca^{2+} binding site and is found in PKCα,β,γ but not in PKCε,δ (Ca^{2+}-independent) or PKCζ (Ca^{2+} and DAG independent), although all isoenzymes retain a PS requirement. The involvement of the C2-domain in the PS unsaturation dependence was tested with a recombinant-PKCε preparation. The results again show a general trend for increased unsaturation decreasing the activity of the PKC. However, it should be noted that sensitivity to unsaturation was diminished considerably, suggesting that the C2-domain of PKC is indeed important in conferring sensitivity to PS unsaturation.

In a previous study that examined the effect of increased PC unsaturation on PKC activity, it was concluded that lipid order, assessed from fluorescence anisotropy measurements of the fluorophore, DPH, was not the major parameter sensed by the PKC. The results in Figure 2 compare the effects of increased PC and PS unsaturation on both PKC activity and on lipid order assessed from DPH anisotropy measurements. The finding of opposite effects indicate that lipid order is unlikely to explain both effects, further indicating that PKC activity is governed by head group interactions rather than those of the acyl chain region.

To further examine the question of what factor might be involved in the effects of PS unsaturation on PKC, a plot of the PS-double bond index and percentage of saturation, as a function of PKC activity, are shown in Figure 3. It can be seen

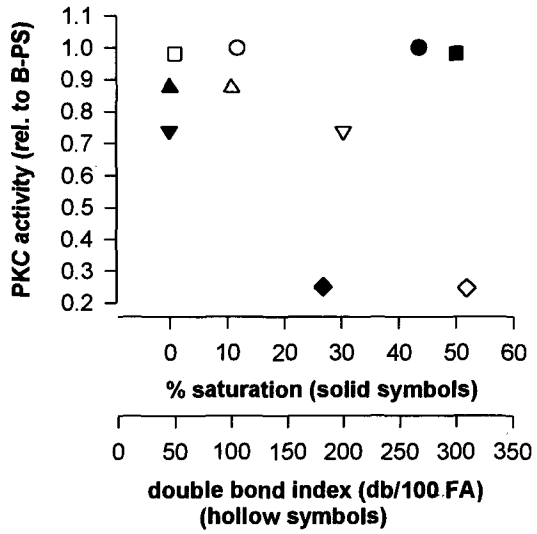


FIG. 3. PKCα activity is not simply related to the % saturation or double bond index. PKCα activity for PS/PC vesicles in which the PS was varied as a function of the % of saturated fatty acids/100 fatty acids or as a function of the number of double bonds/100 fatty acids. Brain-PS (B-PS), ●, ○, POPS, ■, □; DOPS, ▲, △; DLPS, ▼, ▽; K-PS, ◆, ◇. Abbreviations as in Figure 1.

that neither of these parameters was simply related to the effect of PS-unsaturation.

CONCLUSION

Representative PKC isoenzymes of the a and e types were shown to vary in activity as a function of PS unsaturation, activity decreasing with increased unsaturation, opposite to the effect of varied PC unsaturation. The Ca^{2+}-dependent isoform, PKCα, was found to be more sensitive than the Ca^{2+}-independent form, PKCε. It is concluded that the parameters normally used to assess unsaturation cannot explain the effect of varied PS-unsaturation. Therefore, it would appear that, by contrast to the situation with PC (10), PS molecular species interact in a specific manner with PKC. Fluorescence spectroscopic approaches are currently being investigated to elucidate the details of the PS-PKC interaction.

ACKNOWLEDGMENTS

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A Role for Phospholipid Polyunsaturation in Modulating Membrane Protein Function

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ABSTRACT: Visual transduction is one of the best characterized G protein—coupled signalling systems. In addition, about 50% of the disk membrane phospholipid acyl chains are 22:6n-3, making this system ideal for determining the role of polyunsaturation in modulating membrane-signalling systems. The extent of formation of metarhodopsin II (MII), the G protein—activating photointermediate of rhodopsin, was studied in phospholipid vesicles composed of a variety of phosphatidylcholines, differing in their acyl chain composition at the *sn*-2 position. The amount of MII formed increased progressively with the level of acyl chain unsaturation at the *sn*-2 position. The effect of added cholesterol was to reduce the amount of MII formed. The acyl chain packing free volume of the rhodopsin containing lipid vesicles was characterized by a fractional volume parameter f_v derived from measurements of the time-resolved fluorescence anisotropy decay of the hydrophobic membrane probe 1,6-diphenyl-1,3,5-hexatriene. The relationship among *sn*-2 acyl chain unsaturation, cholesterol content, and MII formation is explained on the basis of variation in f_v with bilayer lipid composition and a novel model for the packing of phospholipids containing polyenoic acyl chains, such as 22:6n-3.
Lipids 31, S-193–S-197 (1996).

The fact that organisms exist under conditions of essentially constant temperature and pressure requires that cellular membrane properties, which modulate protein function, must be regulated by membrane composition. Analysis of phospholipid molecular species demonstrates a relatively high degree of heterogeneity within a phospholipid class, which is generally associated with a more diverse acyl chain composition at the *sn*-2 position (1). Neuronal and retinal tissue show the highest content of polyunsaturated acyl chains (2). About 50% of retinal rod outer-segment disk membrane phospholipid acyl chains are 22:6n-3 (1). It is especially interesting that in the disk membrane, the major phospholipid classes, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS), contain high levels of 22:6n-3 acyl chains, whereas minor phospholipids, such as phosphatidylinositol (PI) and phosphatidic acid (PA) contain predominantly 20:4n-6 phospholipids (1). Major phospholipids would be expected to play a structural role, while minor phospholipids might have a more direct functional role, suggesting that 22:6n-3 acyl chains play an important structural role in membranes. The importance of the n-3 polyunsaturated acyl chains is demonstrated by the abnormalities in visual (3) and learning skills (2) associated with a deficiency in n-3 fatty acids and docosahexaenoic acid, 22:6n-3 in particular.

In our studies we have employed the visual transduction pathway as a model system in which to study the effect of phospholipid acyl chain composition on signalling systems (4). The visual pigment, rhodopsin, serves as a prototypical G protein–coupled receptor in this pathway (5). Photoactivation of rhodopsin is analogous to agonist activation of liganded receptors. The visual transduction pathway is well suited for studies to investigate a structural role of polyunsaturated fatty acids for the following reasons: (i) changes in visual response in 22:6n-3–deficient animals are reflected in both abnormal electroretinograms and visual evoked potentials (3); (ii) all three major phospholipid classes in the retinal rod outer segment disk membrane, where rhodopsin is located, contain high levels of 22:6n-3 acyl chains (1); and (iii) the visual transduction system is a G protein–coupled receptor system (5); hence, observations made on this system will be applicable to other G protein–coupled systems.

In this paper, we describe studies in which the extent of formation of metarhodopsin II (MII), the form of photoactivated rhodopsin that activates the rod outer segment G protein, transducin, is correlated with a phospholipid acyl chain–dependent membrane physical property (4), the membrane acyl chain packing free volume. This property is defined as the difference between the measured molecular volume determined from density measurements and the molecular volume determined for the sum of the volume of atoms making up the molecule. Due to disorder and defects in the acyl chain packing, this property will always be positive. A parameter, the fractional volume f_v which is derived from the

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; MI, metarhodopsin I; MII, metarhodopsin II; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinnositol; PS, phosphatidylserine.

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time-resolved anisotropy behavior of the hydrophobic membrane probe 1,6-diphenyl-1,3,5-hexatriene (DPH), is used to characterize the relative acyl chain packing free volume of a variety of compositionally defined lipid bilayers (6). Results of these studies are interpreted in terms of a novel phospholipid acyl chain packing model, which directly addresses the structural role of 22:6n-3 acyl chains in membranes.

EXPERIMENTAL PROCEDURES

Sample preparation. Phospholipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. Butylatedhydroxyltoluene at a level of 1 molecule per 500 phospholipid molecules was added to stock solutions to prevent lipid peroxidation, and all procedures were carried out under argon. Rhodopsin was purified from frozen bovine retinas (Lawson, Inc., Lincoln, NE) *via* Concanavalin A (Pharmacia, Piscataway, NJ) affinity chromatography, and large unilamellar vesicles consisting of lyophilized lipid and purified rhodopsin in a molar ratio of 100:1 (75:1 for di-22:6 PC), respectively, were prepared using a dilution-reconstitution method (7). Total phospholipid content of vesicles was determined by phosphate analysis following the method of Bartlett (8). Samples for rhodopsin photochemical studies were prepared by diluting a vesicle stock solution with the appropriate temperature-compensated buffer (10 mM piperazine-*N,N'-bis*(a-ethanesulfamic acid, 60mM KCl, 30mM NaCl, 2mM MgCl2, 50μM diethylenetriaminepentaacetic acid) to a rhodopsin concentration of 0.25–0.30 mg/mL. Samples for DPH fluorescence studies were prepared by diluting stock solutions to 0.5 mM phospholipid, and DPH was added to level of 1 DPH per 250 lipid molecules.

Equilibrium absorbance measurements. Spectra were acquired with a Hewlett-Packard (Palo Alto, CA) 8452A diode array spectrophotometer (0.2 second measurement yielded < 0.3% bleach by measuring beam). Deconvoluted difference spectra of MI–MII equilibrium mixtures were derived from a series of 4 absorption spectra consisting of (i) unbleached sample; (ii) sample after a controlled exposure to light, which photolyzed 20–25% of the rhodopsin; (iii) sample used to obtain spectrum 2, after addition of hydroxylamine; and (iv) sample used to obtain spectrum 3, after light exposure to photolyze all of the rhodopsin in sample (9). Difference spectra consisting of the contributions from an equilibrium mixture of MI and MII, at 3 s postflash, were constructed by first deriving the spectral band corresponding to the unbleached rhodopsin. Individual MI and MII spectra were deconvoluted from the spectrum of their equilibrium mixture by using nonlinear least squares to fit the sum of two asymmetric Gaussian bands associated MI and MII, to the equilibrium spectral profile (Fig. 1). The equilibrium constant for the MI to MII conversion is given by K_{eq} = [MII]/[MI].

DPH fluorescence depolarization measurements. Fluorescence lifetime and dynamic depolarization measurements were performed at 37, 30, 20, and 10°C. Differential polarization measurements were corrected for all instrumental factors. Rotational depolarization properties were characterized with a model based on the bimodal distribution model for DPH in a lipid bilayer of Ameloot et al. (10). This model characterizes DPH equilibrium orientational properties in terms of a bimodal orientational distribution function, $f(\theta)$ (6). Overall equilibrium ordering experienced by DPH was quantified by the fractional volume parameter f_v which characterizes the volume available for probe reorientational motion relative to that available in an unhindered, isotropic environment, where $f_v = (1/2f(\theta)_{max})\int f(\theta)\sin\theta d\theta$.

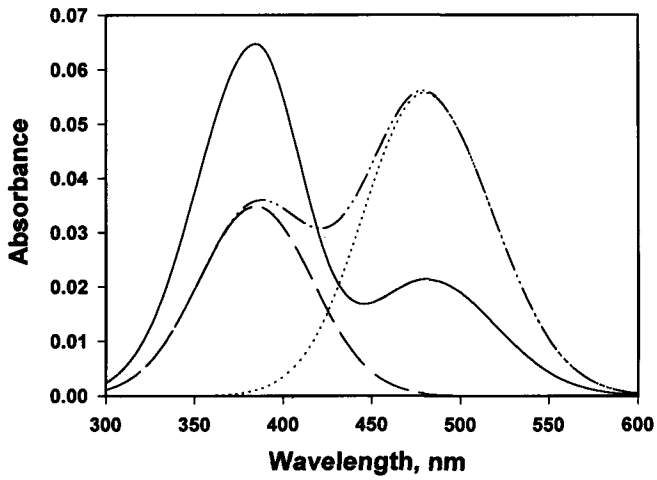


FIG. 1. Examples of the deconvolution of MI and MII spectral contributions. Metarhodopsin I (MI) (••••••), metarhodopsin II (MII) (— —), and combined MI + MII (—— ••• —— •••) spectral contributions for an MI ↔ MII equilibrium mixture in di-14:0 PC. The combined MI + MII (——) spectral contributions for rhodopsin in 16:0,22:6n-3 phosphatidylcholine (PC) are shown for comparison. Both samples were at 37°C. The deconvoluted spectral contribution were obtained using the procedure described in the Experimental Procedures section.

RESULTS

The phospholipid acyl chain dependence of the MI–MII equilibrium is demonstrated in Figure 1, where the deconvoluted spectral contributions of MI and MII for rhodopsin in 16:0,22:6n-3 PC and di-14:0 PC bilayer vesicles are shown. MI and MII have spectral peaks at 478 nm and 380 nm, respectively. Clearly, higher levels of MII formation are observed in the 22:6n-3 containing phospholipid. Data like those shown in Figure 1 allow the determination of the equilibrium constant K_{eq} for the MI–MII conversion. A comparison of K_{eq} for rhodopsin in a variety of compositionally defined phospholipid vesicles is shown in Figure 2. K_{eq} is seen to increase with higher levels of acyl chain unsaturation. The presence of a 22:6n-3 *sn*-2 chain in a mixed-chain phospholipid results in about a six-fold increase in K_{eq} relative to the saturated di-14:0 PC. Relative to both the 22:6n-3 and 20:4n-6 mixed-chain PC, the introduction of a second polyunsaturated chain results in about a two-fold increase in K_{eq}, emphasizing the importance of these chains in promoting MII formation.

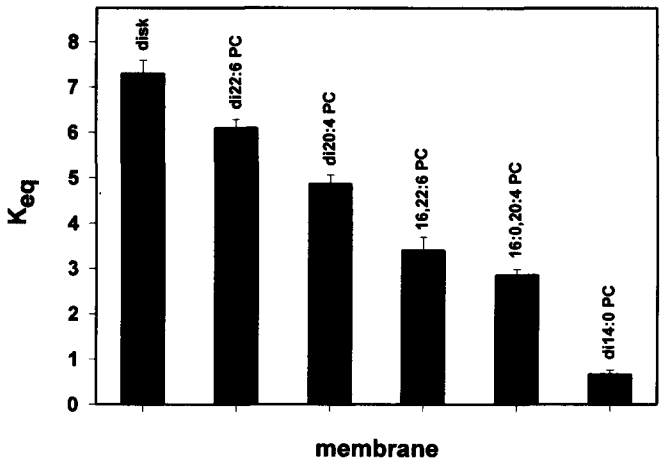


FIG. 2. The compositional dependence of the MI ↔ MII equilibrium constant K_{eq}, determined for rhodopsin in a variety of membranes at 37°C. Abbreviations as in Figure 1.

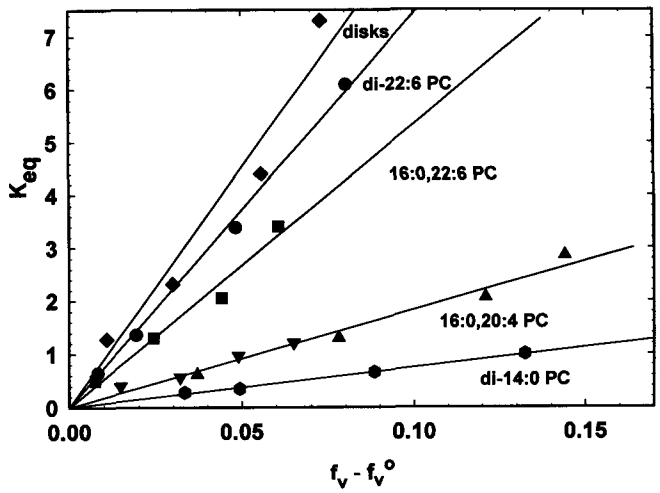


FIG. 3. Correlation between the MI ↔ MII equilibrium constant K_{eq} and the normalized fractional volume f_v—f_v^0, for rhodopsin in disks (◆), di-22:6n-3 PC (●), 16:0,22:6n-3 PC (■), 16:0,20:4n-6 PC (▲), 16:0,20:4n-6 PC containing 30 mole% cholesterol (▲), and di-14:0 PC (×✱✱). The parameter f_v^0 is the fv intercept value of the K_{eq} vs. f_v correlation line. Subtraction of f_v^0 shifts all of the correlation lines to a common origin, making a comparison of the variation in slopes of the correlation lines more direct. Samples contained a ratio of phospholipid to rhodopsin of 100:1, except for di-22:6n-3, which had a 75:1 ratio. Abbreviations as in Figure 1.

In parallel studies of the time-resolved fluorescence anisotropy of DPH, f_v was measured for the same-rhodopsin–containing vesicles. K_{eq}, for several phospholipid compositions and temperatures, is plotted vs. f_v in Figure 3. Here we see that there is a linear correlation between K_{eq} and f_v for each phospholipid and that the slope of the correlation line increases with higher levels of polyunsaturation. This result demonstrates that, at a given value of f_v, increased MII formation occurs in the more unsaturated phospholipids. The effect of having a dipolyunsaturated phospholipid is always to increase K_{eq} substantially above that measured for the mixed-chain phospholipid containing the same acyl chain in only the *sn*-2 position.

Cholesterol is an important component of biological membranes and was shown to decrease acyl chain packing free volume (11). When 30% cholesterol is incorporated into 16:0,20:4n-6 PC bilayers, both K_{eq} and f_v decrease. The resulting correlation points, however, lie on the initial correlation line determined for the cholesterol-free 16:0,20:4n-6 PC vesicles Figure 3. Similar results have been observed for egg PC containing 0, 15, and 30 mole % cholesterol (12).

DISCUSSION

In our studies, we have used the extent of photoactivation of the visual pigment, rhodopsin, as a measure of the ability of a membrane with a defined lipid composition to support optimal protein function. Data presented here demonstrate three important points relative to modulation of rhodopsin photoactivation by phospholipid acyl chain composition. These are: (i) the level of MII formed, i.e., larger values of K_{eq}, increases with the level of unsaturation, a trend that correlates with a concomitant increase in acyl chain packing free volume of the bilayer; (ii) in mixed-chain phospholipids, behavior of the system is correlated with the level of acyl chain unsaturation at the *sn*-2 position; and (iii) although cholesterol reduces both K_{eq} and the acyl chain packing free volume, the K_{eq}–f_v correlation behavior of the system is still determined by the acyl chain at the *sn*-2 position.

A phospholipid acyl chain packing model, which provides an explanation of our results, is shown in Figure 4. Substantial evidence exists to demonstrate that saturated-saturated chain interactions are stronger than unsaturated-unsaturated chain interactions (13,14), suggesting that, in mixed-chain phospholipids like 16:0,22:6n-3 PC, there will be a strong tendency for the phospholipids to pack in a fashion that maximizes *sn*-1 chain interactions. Figure 4A shows a transverse view of the disk membrane, demonstrating the tendency for the *sn*-1 chains to interact with one another. A top view of a cross section through the bilayer midplane is also shown in Figure 4B. *sn*-1 Chain interactions can be maximized by cluster formation in the plane of the membrane, where the interior of the cluster in made up of saturated *sn*-1 chains, and the exterior is composed of *sn*-2 chains. This type of cluster formation is depicted in Figure 4B. Raman vibrational spectroscopic measurements on mixed-chain PC support the existence of these types of structures (15). Conversion of MI to MII is associated with a 100-cc/mole volume change (16,17), indicating that MII has a larger volume than MI. Under such conditions, lateral compressibility of the membrane and the associated acyl chain packing free volume, are expected to play a critical role in determining activation energy for the MI–MII conversion. In the proposed phospholipid packing model, *sn*-2 chains form the interface between domains and hence determine the lateral packing properties of the system. This is consistent with our finding that the slope of the correlation line varies with the substitution at the *sn*-2 position. In addition, evidence in the literature suggests that cholesterol interacts preferentially with saturated acyl chains (13,18) and should therefore interact with *sn*-1 chains. Thus cholesterol could partition into the interior of the clusters and change the lifetime and/or the size of

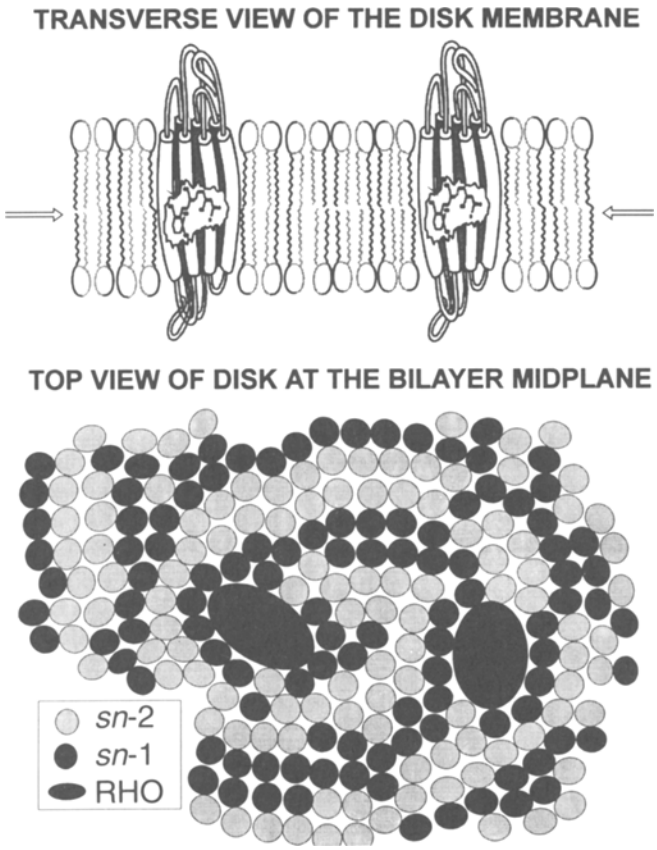


FIG. 4. The microdomain model for acyl chain-packing in a polyunsaturated, mixed-chain phospholipid bilayer containing rhodopsin. (A) Transverse view of the disk membrane showing the phospholipid *sn*-1 (black) and the *sn*-2 (gray) chain packing. (B) Top view of the disk membrane at the bilayer midplane, in which the PC molecules are depicted by pairs of circles representing the *sn*-1 chain (black) and *sn*-2 chain (gray). Rhodopsin is shown surrounded by a rapidly exchanging boundary layer of phospholipid molecules. *sn*-1 Chains are oriented toward the tightly packed interior of the domain, while *sn*-2 chains are oriented toward the domain boundary and are therefore thought to determine the lateral compressibility properties of the bilayer. As the level of unsaturation of the *sn*-2 chain increases, the interactions at the microdomain interfaces will become less cohesive, and the membrane acyl chain—packing free volume will increase, as will the lateral compressibility of the membrane. In the liquid crystalline phase, domains will have a characteristic lifetime and size, which can be influenced by the partitioning of lipid-soluble molecules, such as cholesterol, into the bilayer. Therefore, cholesterol can reduce the free volume by ordering the saturated *sn*-1 chains at the interior of the microdomain, leaving domain-domain interactions unchanged. Abbreviations as in Figure 1.

the clusters, thereby reducing the free volume. The domain interfaces, however, would still be formed by *sn*-2 chains and the lateral packing properties would still be determined by the *sn*-2 chain properties. This is consistent with our finding that, although cholesterol reduces both K_{eq} and f_v in rhodopsin containing 16:0,20:4n-6 PC (5) and egg PC vesicles (13), correlation data points for the cholesterol-containing bilayers fall on the correlation line determined for the cholesterol-free phospholipid vesicles. Our model predicts that in mixed acyl chain phospholipid bilayers, dipolyunsaturated phospholipid molecules, such as di-22:6n-3 PC, will pack between the domains, so as to maintain maximal saturated *sn*-1 chain interactions.

The studies presented here suggest that there are at least two levels whereby lipid composition can regulate membrane properties, which modulate the function of certain classes of liganded receptors. These are primary regulation by phospholipid acyl chain composition and secondary regulation by cholesterol content , the latter being dependent on the presence of a saturated acyl chain. Highly unsaturated phospholipids, such as 16:0,22:6 PC provide the membrane with unique structural properties in the form of microclusters or lateral packing domains. In addition, these phospholipids impart increased acyl chain packing free volume to the bilayer, which promotes formation of MII, the active receptor form of rhodopsin. While the level of MII formation in di-22:6n-3 PC and 16:0,22:6n-3 PC are among the highest observed in the pure phospholipid vesicle systems, the highest values of K_{eq} are observed in the disk membrane, which is a complex mixture of varied head groups and acyl chains. Further studies will be required to assess relative effects of different phospholipid classes and mixed molecular species on properties of bilayers associated with modulation of membrane protein function.

Our findings that bilayers containing lower levels of acyl chain unsaturation have reduced levels of MII formation suggest that the diminished amplitude in the α-wave of the electroretinogram observed whole in animals, which are deficient in phospholipids containing 22:6n-3 (2,3), is associated with decreased levels of photoactivated rhodopsin. Visual transduction is a prototypical G protein–coupled signalling system, where rhodopsin serves as the receptor. Therefore conclusions drawn from the present studies, relative to the enhanced formation of the active receptor conformation in 22:6n-3–containing phospholipids, should be applicable to other G protein–coupled systems, as well as receptor systems where the agonist binding site is within the bilayer and composed of several transmembrane spans.

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Nuclear Magnetic Resonance Investigation of Hydrocarbon Chain Packing in Bilayers of Polyunsaturated Phospholipids

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ABSTRACT: ^{2}H nuclear magnetic resonance (NMR) on chain-deuterated phospholipids has been used to study the influence of the degree of unsaturation on lipid chain packing and on area per molecule at the lipid water interface. Order and motions of deuterated stearic acid in position sn-1 of phosphatidylcholines (PC) containing 18:0, 18:1n-9, 18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3, or 22:6n-3 in position sn-2 were investigated in pure PC and in mixtures of PC in a phosphatidylethanolamine (PE) matrix. Results reveal that lipid packing in bilayers is mainly controlled by packing requirements at the lipid water interface. Increasing degrees of unsaturation lower chain order and increase area per PC molecule, whereas inclusion of PE in model membranes has the opposite effect. Chain order and motions in highly unsaturated lipid membranes are less sensitive to changes in temperature. Temperature sensitivity decreases further upon incorporation of PC into a PE matrix. Unsaturation induces chain disordering, which may be interpreted as an increase in area per molecule of lipids toward the center of the bilayer. This may result in a lower packing density of unsaturated lipids at the lipid water interface. We hypothesize that these differences in lipid packing and dynamics may influence activity of membrane proteins.

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Activity of membrane-associated proteins such as rhodopsin (1–3), protein kinase C (4), calcium ATPase (5,6), apocytochrome C (7), and cytochrome b_5 (8), is sensitive to head group structure and unsaturation of hydrocarbon chains of lipid bilayers. Cells carefully control head group composition and chain unsaturation to maintain optimal conditions for cell function (9). Part of the observed correlations between lipid composition and membrane function are likely due to the biophysical properties of lipid membranes.

Attempts to link protein function to structure and dynamics of lipid bilayers have a long history. The fluid mosaic model of biological membranes highlighted the importance of a liquid crystalline lipid environment for membrane proteins (10). Unsaturation provides the appropriate fluidity for membrane proteins. The existence of a boundary layer of lipid around proteins had been suggested, with a preference for some lipid classes. In their mattress model of lipid bilayers, Mouritsen and Bloom (11) focused on the energy of a mismatch in hydrophobic thicknesses of lipid bilayers and membrane-spanning proteins. A departure from matching, e.g., as a result of differences in membrane composition, resulted in a mechanical strain in the membrane. Gruner, *et al.* (12,13) proposed that differences in spontaneous curvature of membranes result in lipid-packing stress in bilayers. Spontaneous curvature of membranes has been shown to depend on unsaturation and head group structure. Head groups and unsaturation also influence area per molecule at the lipid water interface and lateral compressibility of membranes (14). Dratz and Holte (15) introduced the molecular spring model to explain specific requirements of rod outer segment disk membranes for docosahexaenoic acid. They proposed that this sixfold unsaturated fatty acid can contract and expand with a minimal input of energy, allowing the lipid bilayer to thicken upon formation of metarhodopsin II. Using ^{2}H nuclear magnetic resonance (NMR) measurements, Holte (16) showed that lipid bilayers containing docosahexaenoic acid increased in thickness by 0.6 Å when rhodopsin formed metarhodopsin II. Straume and Litman (17) measured a fractional volume available for reorientation of fluorescence probes in membranes and demonstrated a correlation between free volume and metarhodopsin II formation. Despite many efforts, the mechanism by which lipids control protein function are not yet known. Experimental data describing bilayer structure with better resolution are warranted.

The primary tools for obtaining information about dimensions of lipid bilayers are X-ray and neutron diffraction methods (18–24). Unfortunately, the biologically relevant liquid crystalline phase of lipid water dispersions is characterized by a low degree of long-range order, which results in significant uncertainty in determination of parameters like membrane thickness and area per lipid molecule. A typical value for the resolution of membrane thickness measurements is ±1 Å, which translates into a resolution of ±2 Å^2 for area per molecule. A change in area per molecule of this order translates into an energy per lipid of about $7.5 \cdot 10^{-3}$ kT (4.5 cal/mol) (25). If we consider that every protein interacts with

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Abbreviations: DOPE, dioleoylphosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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100 lipid molecules in a domain or cluster, then this experimental uncertainty translates into 0.75 kT per molecule of protein (450 cal/mol). This energy must be compared with energies that shift membrane protein transitions, e.g., metarhodopsin I–metarhodopsin II equilibrium, which are of the order of 0.1 kT (60 cal/mol) (26). The crude comparison indicates that the current precision of diffraction methods may be insufficient to resolve small changes in membrane structure that are relevant for lipid protein interaction.

^{2}H NMR on deuterated lipids yields an order parameter profile of lipid hydrocarbon chains (27), which has been shown to be very sensitive to changes in thickness and area per molecule (28). While diffraction methods operate on a timescale of 10^{-11} s and shorter, measurement of deuterium order parameters has an intrinsic timescale of 10^{-5} s. Over this period of time, all lipid molecules sample every chain conformational state available in a typical bilayer, resulting in well-resolved NMR signals that are sensitive to very small changes of lipid order. The spectral resolution translates into a sensitivity for changes of bilayer thickness of ±0.2 Å and changes in area per molecule of ±0.4 Å^2. This corresponds to an energy change per lipid on the order of $3 \cdot 10^{-4}$ kT (0.18 cal/mol) or an energy of 0.03 kT (18 cal/mol) per protein molecule in a domain or cluster of 100 lipids, which is well within the range of physiologically relevant energy shifts. Thus, NMR has a sensitivity better suited for observing small changes in membrane structure that are relevant for membrane proteins, although interpretation of NMR data in terms of geometrical parameters of a bilayer is less straightforward than interpretation of diffraction data. We have used order parameter profiles of saturated *sn*-1 chains to characterize the thickness and cross-sectional area of lipid bilayers formed from lipids with 1–6 double bonds in the sn-2 position. The influence of the smaller PE head group has been studied as well. Results are combined here to describe the influence of unsaturation and head group on lipid packing.

MATERIALS AND METHODS

Unsaturated lipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and, when necessary, purified by preparative reverse-phase high-performance liquid chromatography at ambient temperature with a column packed with Adsorbosphere C18 HS 7U (Alltech Assoc., Inc., Deerfield, IL). Analytical grade methanol was used as a mobile phase. The antioxidant butylated hydroxytoluene was added at a ratio of lipid to butylated hydroxytoluene of 250:1 (mol/mol). Lipid mixtures were prepared in organic solvents. About 5 mg deuterated PC and 20 mg PE were used per sample. Solvents were removed under a stream of argon and in vacuum. Lipids were hydrated with 50 wt% deuterium-depleted water and sealed in argon-filled glass ampoules. Spectra were acquired on a Bruker MSL 300 spectrometer (Billerica, MA) with a quadrupolar echo sequence (29) using a delay time of 30 μs between pulses and a pulse repetition rate of 0.5 s. Typically, 8,196 scans were accumulated using a dwell time of 1 μs. Smoothed order parameter profiles were calculated following the method of Lafleur *et al.* (30).

RESULTS AND DISCUSSION

Introduction of the first double bond into an 18-carbon chain of PC has been shown to reduce the phase transition temperature between gel and liquid crystalline lamellar phases by more than 50°C. Among the unsaturated PC, the transition temperature of (18:0)(18:2n-6) PC was lowest (31,32). In Figure 1, typical ^{2}H NMR spectra for phospholipid water dispersions and calculated order parameter profiles are given. For pure PC and for PC in dioleoylphosphatidylethanolamine (DOPE), chain order decreases with an increasing degree of unsaturation. Changes are largest between mono- and diunsaturated lipids. Differences between lipids with 3–6 double bonds at 30°C are smaller than the experimental error. Reduction of chain order corresponds to a decrease in hydrophobic thickness of lipid bilayers of approximately 0.7 Å. In combination with the increased volume of highly unsaturated chains vs. monounsaturated chains (22 carbon atoms vs. 18), this translates into an increase in area per molecule for polyunsaturated vs. monounsaturated PC of about 5 Å^2. PC in a DOPE matrix at a molar ratio of 1:5 have significantly larger quadrupolar splittings and thus greater order of their *sn*-1 hydrocarbon chains than do bilayers of pure PC (see Figs. 1 and 2). The DOPE matrix induced the largest ordering effect on

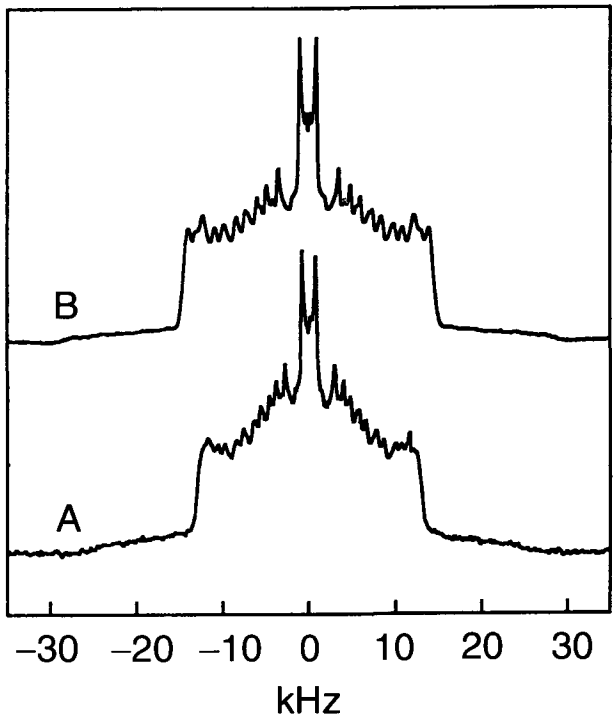


FIG. 1. ^{2}H NMR spectra for (18:0_{d35})(22:6n-3)PC at 30°C. (A) phosphatidylcholine (PC) dispersion; (B) PC incorporated into a matrix of dioleoylphosphatidylethanolamine (DOPE) (molar phosphatidylethanolamine/PC ratio = 5:1).

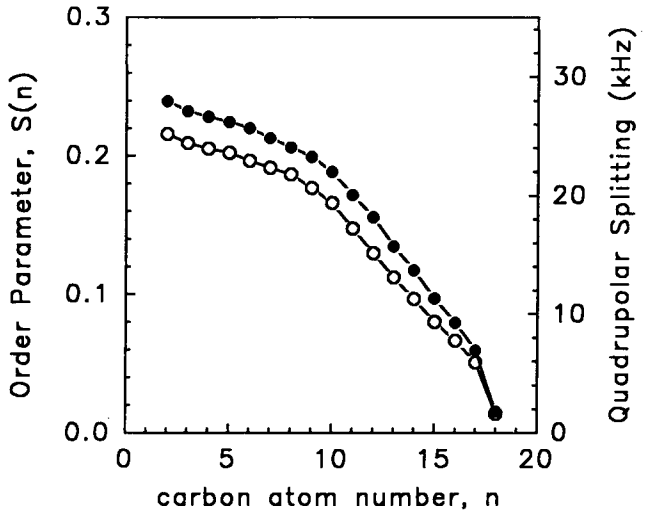


FIG. 2. Order parameter profiles of perdeuterated stearic acid calculated from spectra in Figure 1: ○ (18:0_{d35})(22:6n-3)PC; ● (18:0_{d35})(22:6n-3)PC in matrix of DOPE. The smaller PE head group compresses hydrocarbon chains of PC molecules laterally resulting in a significant increase of chain order (see Fig. 1 for abbreviations).

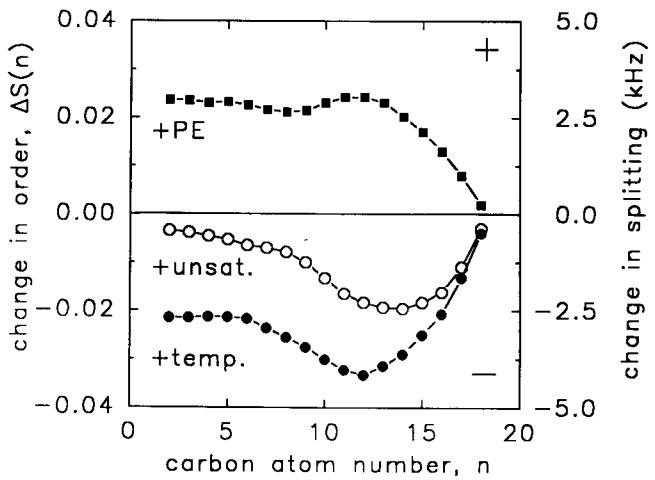


FIG. 3. Difference order parameter profiles, ΔS(n) , of perdeuterated stearic acid as a function of unsaturation, temperature, and head group composition: ○ ΔS(n) between (18:0_{d35})(22:6n-3) PC and (18:0_{d35})(18:1n-9) PC at 37°C; ● ΔS(n) of (18:0_{d35})(18:1n-9) PC between 27°C and 47°C; ■ ΔS(n) between (18:0_{d35})(22:6n-3) PC in a matrix of DOPE and pure (18:0_{d35})(22:6n-3)PC at 30°C (see Fig. 1 legend for abbreviations).

fully saturated distearoylphosphatidylcholine, while unsaturated PC appear to have significantly less lateral compressibility. Difference in order translates into an increase of hydrocarbon thickness of PE/PC vs. PC bilayers of about 1 Å, equivalent to a decrease in area per molecule of the order of 2 $Å^2$. Similar changes have been observed for chain-perdeuterated dimyristoylophosphatidylcholine (DHPC-d_{54}) incorporated into a DOPE matrix (33).

The magnitude of decrease in order along the lipid hydrocarbon chain as a result of double bonds in position *sn*-2 is largest approximately two-thirds of the way down the chain (Fig. 3). This suggests an inhomogeneous increase in area per molecule that is greatest toward the center of the bilayer (32). Such a change may indicate a wedge-shaped conformation for the acyl chain, which results in a looser packing of polyunsaturated lipids at the lipid water interface. It may explain the deeper penetration of solvent molecules into membranes containing unsaturated lipids.

The lipid hydrocarbon chain order decreases with increasing temperature (Fig. 3). Decreased chain order translates into thinner membranes and a larger cross-sectional area per molecule. Change in order as a function of temperature can be approximated by a quadratic function with a dominating linear term. Linear temperature coefficients for polyunsaturated lipids are nearly two times smaller than those of monounsaturated lipids. Incorporation of the PC into a DOPE matrix results in a reduction of the temperature coefficient of saturated and monounsaturated PC by a factor of 0.7. Both the introduction of double bonds and the presence of PE in the lipid matrix reduce the sensitivity of chain order to temperature.

The effect of unsaturation on chain order is often compared with the effect of temperature changes. Both double bonds and higher temperatures result in a decrease of chain order (Fig. 3). Comparison of order parameter profiles, however, reveals that increasing temperature lowers chain order along the entire hydrocarbon chain, while unsaturation lowers chain order mostly in the center of the bilayer. This illustrates that biophysical properties of polyunsaturated membranes are *not* equivalent to properties of less unsaturated membranes investigated at a higher temperature.

Area per lipid molecule is maintained between rather narrow limits to minimize contact between water and hydrophobic regions of the lipid (34). Free energy of the lipid water interface is the major factor that determines packing of lipids in bilayers. Bulkier PC head groups and multiple double bonds in the *sn*-2 chain of the lipids result in an increase in area at the lipid water interface. While unsaturation changes the interface through repulsive forces in the hydrophobic core, area increase induced by head group methylation is the result of changes in polar interaction and hydration. Although both interactions increase area per lipid molecule, their influence on the spontaneous radius of curvature of membranes is of opposite sign: unsaturation and the smaller PE head group favor formation of inverse hexagonal or cubic phases with highly curved monolayers, while the bulkier PC head group stabilizes the lamellar phase (35). This underscores the existence of a tight link between the hydrophobic core and the head group region for lipid packing.

How may we relate bilayer properties due to acyl chain unsaturation and head group size to lipid-protein interactions? (i) We hypothesize that the efficiency of other molecules to penetrate a bilayer is related to the loosening of lipid packing at the interface as a result of unsaturation and head group methylation. This may explain why membrane binding of some proteins depends on unsaturation and the PE/PC ratio. (ii) Unsaturation and head group size change the propensity

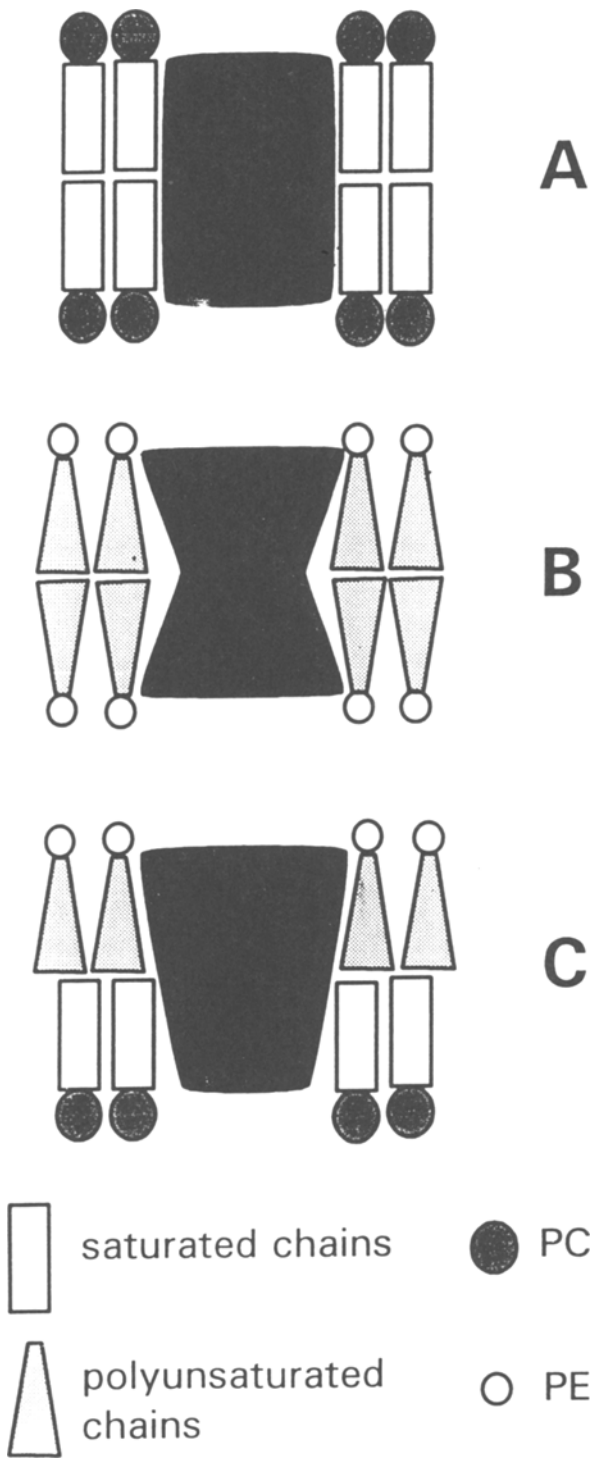


FIG. 4. Schematic representation of molecular packing of a transmembrane protein in bilayers composed of lipids with different head group size and degree of unsaturation. Lipid molecules with small head groups and polyunsaturated hydrocarbon chains accommodate protein transitions into states in which transmembrane helices are tilted (B and C). Membranes with an asymmetric distribution of lipids between monolayers may even accommodate protein structures in which the hinge region of tilt is located outside the bilayer center (C).

of lipid monolayers for curvature and therefore have an influence on membrane curvature stress in a lamellar arrangement. Changes in the arrangement of transmembrane helices during structural transitions of proteins may be favored by lipids that prefer a particular curvature (Fig. 4). This may apply to structural transitions of membrane-bound rhodopsin. Liebman *et al.* (36) suggested that the meta I–meta II transition results in an increase in splay of the 7 transmembrane helices. Tilted helices will create curvature stress in the lipid bilayer around the protein. Depending on the location of the hinge region of protein tilt, this curvature stress may differ between the monolayers. In at least one monolayer, lipids that prefer a smaller area per molecule at the lipid water interface would support the transition. This may explain why polyunsaturated lipids with the smaller PE head group favor formation of the meta II state of rhodopsin (37). (iii) Introduction of polyunsaturated fatty acids and of PE head groups into membranes reduces thermal area expansivity coefficients of membranes. The smaller PE head group reduces area per molecule at the lipid water interface and compresses hydrocarbon chains. Our results demonstrate that saturated PC are more compressed by the DOPE matrix than polyunsaturated PC, suggesting that the elastic area compressibility modulus of polyunsaturated phospholipids is higher. The meta I–meta II transition of rhodopsin has been linked to lateral expansion of rhodopsin, which may be sensitive to differences in membrane compressibility. (iv) There is evidence that proteins may interact specifically with phospholipids (38).

The data provide several mechanisms by which membrane protein function can be modulated by lipids. The small changes in membrane structure related to altered protein activity can be detected by extremely sensitive techniques only, and ^2H NMR spectroscopy is ideally suited for this requirement.

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Marked Enrichment of the Alkenylacyl Subclass of Plasma Ethanolamine Glycerophospholipid with Eicosapentaenoic Acid in Human Subjects Consuming a Fish Oil Concentrate

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ABSTRACT: Alteration in human platelet fatty acid levels with the consumption of fish oils containing eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) have been well documented, but changes in the fatty acid composition of plasma plasmalogenic phospholipid under similar circumstances have not been delineated. In the present study, subjects consumed the fish oil concentrate (MaxEPA) for 6 wk followed immediately by a 6-wk recovery period with no fish oil ingestion. Plasma total choline glycerophospholipid (GPC) and ethanolamine glycerophospholipid (GPE) subclasses isolated from blood samples obtained at 0, 3, 6, 9 and 12 wk of the experimental period were analyzed for fatty acid composition via thin-layer and gas-liquid chromatographic techniques. Consumption of fish oil for 3 or 6 wk significantly elevated the content of n-3 fatty acids while concomitantly decreasing n-6 fatty acid levels in plasma total GPC and in diacyl and alkenylacyl (plasmalogen) GPE. Alkenylacyl GPE exhibited the greatest alteration of both n-3 and n-6 fatty acid levels. Following 6 wk of supplementation with fish oil, EPA rose by 24.6 mol% in alkenylacyl GPE compared to increases of 6.7 and 7.1 mol% in diacyl GPE and total GPC, respectively. The increase in EPA (from 5.0 to 29.6 mol%) in plasma alkenylacyl GPE represents amongst the highest enrichment of EPA in any lipid yet reported in human subjects. DHA also rose by 8.0, 4.8, and 3.1 mol% in alkenylacyl GPE, diacyl GPE, and total GPC, respectively. Alkenylacyl GPE exhibited the greatest mol% decline (by 18.7 mol%) in arachidonic acid (AA, 20:4n-6) following 6 wk of fish oil supplementation. The corresponding decreases of AA in diacyl GPE and total GPC were 8.7 and 1.8 mol%, respectively. Following the 6 wk recovery period, n-3 and n-6 fatty acid levels had returned to pre-supplementation values. The marked enrichment of alkenylacyl GPE in n-3 fatty acids, especially EPA, may be of significance with respect to a unique role for this plasma phospholipid subclass in attenuating certain lipoprotein-mediated cardiovascular effects as observed with fish/fish oil consumption.

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GPC, choline glycerophospholipid; GPE, ethanolamine glycerolphospholipid; LA, linoleic acid; TLC, thin-layer chromatography.

Consumption of fish or fish oils containing n-3 fatty acids appears to offer protective effects against cardiovascular disease. Decreased arterial thrombosis may relate in part to the reduced platelet reactivity observed in fish/fish oil consumers (1,2). Accumulation of n-3 fatty acids, particularly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), in platelet phospholipids following fish/fish oil ingestion has been extensively documented (1,2). In particular, the alkenylacyl (plasmalogen) subclass of ethanolamine glycerophospholipid (GPE) represents one of the major forms of arachidonic acid (AA) containing phospholipid in platelets (3) and, with fish oil feeding, exhibits the greatest alteration in fatty acid composition among human platelet phospholipids (4,5). Total plasma phospholipid n-3 fatty acid levels have also been shown to reflect fish consumption (6,7). We have previously shown that upon supplementation with flax or canola oils containing α-linolenic acid, plasma EPA levels are most significantly altered in the alkenylacyl GPE subclass (8), indicating that this subclass is a sensitive indicator of n-3 fatty acid intake. The enrichment of n-3 polyunsaturated fatty acids in choline glycerophospholipid (GPC) and the subclasses of plasma GPE upon human consumption of fish or fish oil containing high levels of EPA and DHA, however, has not been investigated.

GPC (predominantly diacyl species) is the major phospholipid fraction present in human total plasma or serum at approximately 70% of the total, while GPE comprises about 3% of the total plasma/serum phospholipids (9–11). Similar phospholipid compositions have been reported for the individual lipoproteins in plasma (12). GPC and GPE in human plasma/serum can be fractionated into diacyl and ether-containing subclasses with the alkenylacyl GPE subclass representing about 60–70% of total GPE (9–11). Myher *et al.* (9) have reported on the fatty acid composition of the individual subclasses of plasma phospholipids, including alkenylacyl GPE. However, the effect of fish/fish oil supplementation on the fatty acid compositions of these subclasses in human plasma have not been investigated. The purpose of the present study was to analyze the alterations in fatty acid compositions

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TABLE 1
Fatty Acid Composition of Plasma GPC[a]

Fatty acid	Week 0	Week 3	Week 6	Week 9	Week 12
16:0	31.1 ± 0.6	33.3 ± 0.8	31.0 ± 0.5	33.2 ± 1.8	32.6 ± 0.8
16:1	0.7 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.2
18:0	12.5 ± 0.3^{a}	13.6 ± 0.3^{b,c}	14.4 ± 0.3^{b}	12.5 ± 0.4^{a,c}	12.6 ± 0.1^{a}
18:1n-9	13.3 ± 0.4	11.7 ± 0.9	11.1 ± 0.6	12.6 ± 1.0	12.6 ± 0.7
18:2n-6	23.5 ± 2.1^{a}	16.5 ± 1.3^{b}	16.5 ± 2.0^{b}	23.2 ± 1.4^{a}	24.6 ± 1.5^{a}
20:2n-6	0.5 ± 0.0^{a}	0.2 ± 0.0^{b}	0.3 ± 0.0^{b}	0.5 ± 0.1^{a,b}	0.4 ± 0.1^{a}
20:3n-6	2.6 ± 0.3^{a}	1.1 ± 0.1^{b}	1.0 ± 0.1^{b}	2.8 ± 0.4^{a}	2.8 ± 0.3^{a}
20:4n-6	7.9 ± 1.3^{a}	6.5 ± 0.6^{a,b}	6.1 ± 0.8^{a,b}	6.0 ± 0.9^{b}	6.8 ± 0.9^{a}
20:5n-3	0.8 ± 0.3^{a}	7.4 ± 0.3^{b}	7.9 ± 0.7^{b}	1.0 ± 0.1^{a}	0.7 ± 0.2^{a}
22:5n-3	0.6 ± 0.1^{a}	1.2 ± 0.2^{b,c}	1.4 ± 0.1^{b}	0.8 ± 0.1^{a,c}	0.6 ± 0.1^{a}
22:6n-3	2.8 ± 0.6^{a}	5.2 ± 0.6^{b}	5.9 ± 0.6^{b}	3.3 ± 0.5^{a}	2.4 ± 0.3^{a}

[a]Subjects (n = 5) consumed MaxEPA for a 6-wk period followed by a 6-wk recovery period. At week 0 and after 3, 6, 9, and 12 wk, plasma choline glycerophospholipid (GPC) was analyzed for fatty acid composition. Results are given in mol% as mean ± SEM. Values across each row having different superscripts are significantly different from each other ($P < 0.05$).

of the alkenylacyl and diacyl subclasses of GPE in human plasma, as well as GPC, following the consumption of a fish oil concentrate, including the reversal of these changes following a recovery period in which no fish oil was consumed.

SUBJECTS AND METHODS

Subjects. The experimental protocol was approved by the Human Subjects Committee of the University of Guelph. Five healthy male subjects with a mean age of 26.4 years and a mean weight of 72.7 kg provided informed written consent. The subjects consumed 20 capsules of MaxEPA (Seven Seas Health Care Ltd., Marfleet, United Kingdom), a fish oil concentrate, for 6 wk at regular meal times. This provided for an intake of 3.6 g EPA and 2.4 g DHA per day. The supplementary period was followed by a recovery period of 6 wk in which no MaxEPA was consumed. During the entire experimental period and for 2 wk prior to the start, subjects were asked to refrain from consuming any seafood.

Plasma lipid isolation. At week 0, and after 3, 6, 9, and 12 wk of the experimental period, blood samples were drawn from an antecubital vein into siliconized Vacutainer tubes (Becton-Dickinson, Rutherford, NJ) containing 1/6 volume of an acid-citrate-dextrose anticoagulant (13). The blood was centrifuged at 190 x g for 15 min at room temperature to obtain the platelet-rich plasma. This was removed and centrifuged at 2000 x g for 15 min at room temperature to obtain platelet-poor plasma. Plasma lipids were extracted from 1 mL aliquots of platelet-poor plasma by the method of Folch *et al.* (14).

Separation of plasma phospholipids by thin-layer chromatography (TLC). The plasma lipid extract was applied to Merck silica gel 60 plates (BDH Chemicals Canada Ltd., Toronto, Ontario, Canada) and separated into individual phospholipid classes using a modification of the Mitchell *et al.* (15) two-dimensional TLC system as we have previously described (5). After development in the first dimension, an area of the thin-layer plate containing total GPE was exposed to concentrated HCl fumes for 10 min at a distance of 5 cm. This procedure cleaved the 1-position aldehyde group of alkenylacyl GPE producing lyso GPE acylated in the 2-position (5), which was then separated from diacyl GPE in the second dimension. After separation by TLC, the phospholipids were visualized under ultraviolet by spraying the plate with 2′-7′-dichlorofluorescein (Sigma Chemical Co., St. Louis, MO) in methanol and water (1:1, vol/vol) and exposing to ammonia vapor.

The area of the plate referred to as GPC contains the diacyl, alkenylacyl, and alkylacyl classes, which have been shown to represent 93, 3.6, and 3.4% respectively, of the total GPC in plasma (9). The lyso GPE fraction of the plate contains fatty acid acylated in the 2- position which was obtained from the acid hydrolysis of alkenylacyl GPE. Alkenylacyl GPE represents approximately 60–70% of total GPE in plasma (9–11). The area referred to as 'diacyl GPE' contains mostly diacyl GPE plus some minor amount of alkylacyl GPE. These latter two GPE classes have been documented to account for about 20–40 (9–11) and 8% (9), respectively, of total plasma GPE.

Fatty acid analysis. The GPC, diacyl GPE, and lyso GPE (from alkenylacyl GPE hydrolysis) fractions of the TLC plate were transmethylated in the presence of the internal standard, monopentadecanoin (Nu-Chek-Prep Inc., Elysian, MN), for 2 h at 80°C. The derived fatty acid methyl esters were extracted with petroleum ether, dried under nitrogen, redissolved in hexane and analyzed on a Hewlett-Packard 5890 gas-liquid chromatograph containing a DB-225 megabore column (Chromatographic Specialties Inc., Brockville, Ontario, Canada). Fatty acids were identified by comparison of peak retention times with those of known standards (Nu-Chek-Prep). Correction for any background contamination was achieved by subtracting values obtained from blank regions of the TLC plates.

Statistical analysis. Results were analyzed using a block design, with individual subjects as the blocks. Significant differences in fatty acid levels were determined by analysis of variance followed by Tukey's Studentized range test (17).

RESULTS

Tables 1, 2, and 3 show fatty acid compositions of plasma total GPC, diacyl GPE and alkenylacyl GPE, respectively, during the 6 wk of the supplementary period followed by a 6 wk recovery period. The week 0 (initial) fatty acid composi-

TABLE 2
Fatty Acid Composition of Plasma Diacyl GPE[a]

Fatty acid	Week 0	Week 3	Week 6	Week 9	Week 12
16:0	12.7 ± 0.9	13.2 ± 0.5	13.0 ± 1.7	13.2 ± 0.7	12.2 ± 0.9
18:0	25.3 ± 0.4	24.5 ± 0.7	25.2 ± 0.8	25.0 ± 0.4	23.8 ± 1.5
18:1n-9	12.3 ± 0.8	9.5 ± 0.9	11.9 ± 2.0	11.0 ± 0.8	11.9 ± 0.7
18:2n-6	13.2 ± 1.9^{a}	6.7 ± 1.1^{b}	9.7 ± 2.9^{a,b}	13.7 ± 1.4^{a}	13.7 ± 2.2^{a}
20:3n-6	1.1 ± 0.1^{a}	0.5 ± 0.9^{b}	0.5 ± 0.1^{b}	1.4 ± 0.1^{a,c}	1.7 ± 0.2^{c}
20:4n-6	17.3 ± 1.6^{a}	10.3 ± 1.0^{b,c}	8.6 ± 0.8^{c}	13.5 ± 1.2^{a,b}	14.0 ± 2.0^{a}
20:5n-3	1.1 ± 0.3^{a}	7.4 ± 0.6^{b}	7.8 ± 0.7^{b}	1.4 ± 0.1^{a}	1.3 ± 0.3^{a}
22:5n-3	1.9 ± 0.1^{a}	2.5 ± 0.2^{b}	2.1 ± 0.1^{a,b}	2.0 ± 0.1^{a,b}	1.6 ± 0.1^{a}
22:6n-3	11.0 ± 1.7^{a,b}	19.1 ± 1.9^{c}	15.8 ± 1.9^{a,c}	12.0 ± 0.6^{a,b}	9.3 ± 1.4^{b}

[a]Subjects (n = 5) consumed MaxEPA for a 6-wk period followed by a 6-wk recovery period. At week 0 and after 3, 6, 9, and 12 wk, plasma diacyl ethanolamine glycerophospholipid (GPE) was analyzed for fatty acid composition. Results are given in mol% as mean ± SEM. Values across each row having different superscripts are significantly different from each other ($P < 0.05$).

tions of all three plasma phospholipid classes were similar to previously published data (9–11). Supplementation with the fish oil concentrate, MaxEPA, caused an enrichment of plasma GPC, diacyl GPE, and alkenylacyl GPE in n-3 fatty acids, particularly EPA and DHA. EPA rose to 7.9, 7.8, and 29.6 mol% of fatty acids in total GPC, diacyl GPE, and alkenylacyl GPE, respectively, by the end of the supplementation period, while the corresponding values for DHA were 5.9, 15.8, and 27.6 mol%, respectively. The total increase in n-3 fatty acids in each phospholipid after 6 wk of fish oil supplementation was mostly accounted for by an increase in EPA (57–74% of total increase) followed by DHA (24–41% of total increase). This was also reflected in the molar ratio of EPA/DHA which increased in each of the plasma phospholipids from week 0 to week 6 and then returned to week 0 levels following the recovery period.

Concomitant with the increase in n-3 fatty acids in plasma phospholipids with 6 wk of fish oil supplementation was a decrease in n-6 fatty acid levels. Comparison of week 6 and week 0 values reveals that the individual phospholipid total mol% changes in n-3 and n-6 fatty acids were approximately equal. In plasma total GPC, linoleic acid (LA, 18:2n-6) accounted for the majority (65%) of the total decline in n-6 fatty acids with AA, 20:4n-6 responsible for just over 15% of the decrease (Table 1). In contrast, AA represented about 65% and LA about 28% of the fall in total n-6 fatty acids in both GPE classes studied (Tables 2 and 3). During the recovery period (weeks 6 to 12), fatty acid mol% values decreased (n-3 fatty acids) or increased (n-6 fatty acids) so that they were not significantly different from the pre-supplementation values (week 0) by the end of the recovery period.

At entry (week 0) n-3 fatty acid mol% values were greatest in alkenylacyl GPE compared to both diacyl GPE and total GPC. Alkenylacyl GPE was especially enriched in DHA at 19.6 mol% compared to 11.0 and 2.8 mol% in diacyl GPE and total GPC, respectively. EPA comprised 5.0, 1.1, and 0.1 mol% of fatty acids in alkenylacyl GPE, diacyl GPE, and GPC, respectively, at week 0. Similar differences for EPA and DHA in plasma alkenylacyl GPE relative to diacyl GPE and total GPC of unsupplemented subjects was observed by Myher *et al.* (9). The greatest mol% rise in n-3 fatty acids over the 6 wk supplementary period also occurred in alkenylacyl GPE. Total n-3 fatty acids increased by 33.1 mol% in alkenylacyl GPE, while in diacyl GPE and GPC the increases were 11.3 and 11.0 mol%, respectively. The 33.1 mol% increase in total n-3 fatty acids in alkenylacyl GPE was accounted for by a rise in EPA (from 5.0 to 29.6 mol%), DHA (from 19.6 to 27.6 mol%) and docosapentaenoic acid (22:5 n-3, from 4.7 to 5.2 mol%).

Similar to the n-3 fatty acids, alkenylacyl GPE had the highest AA mol% at week 0 (39.4 mol% versus 17.3 and 7.9 mol% in diacyl GPE and GPC, respectively), and displayed the largest mol% decline in AA with fish oil supplementation (decline of 18.7 mol% vs. 8.7 and 1.8 mol% in diacyl GPE and GPC, respectively). In terms of percentage changes in fatty acids between week 0 and week 6, alkenylacyl GPE and diacyl GPE had similar increases in EPA and DHA and a similar decline in AA. EPA and DHA rose by 6–7-fold and 1.4-fold, respectively, and AA was approximately halved in both subclasses of GPE examined.

DISCUSSION

In this study, we report on the enrichment of human plasma phospholipids (including alkenylacyl GPE) with n-3 fatty acids following supplementation with a fish oil concentrate. Increases of n-3 fatty acids in platelet phospholipids (particularly alkenylacyl GPE) have been reported following the ingestion of fish oil (5). In this study, the level of EPA approached or surpassed the level of AA in all three plasma phospholipids studied following fish oil supplementation. This contrasts with platelet data where AA levels were far greater than EPA in each individual phospholipid despite the consumption of identical amounts of the same fish oil concentrate (5). In plasma, we found a very high enrichment of n-3 fatty acids in the alkenylacyl GPE subclass. This pronounced enrichment of EPA (to 29.6 mol%) in plasma alkenylacyl GPE is, to our knowledge, the highest enrichment of EPA in any lipid yet reported in human subjects upon supplementation with fish oil. Alkenylacyl GPE displayed the greatest alteration in n-3 and n-6 fatty acids (especially EPA and AA, respectively) following the 6 wk of supplementation with MaxEPA.

Whether this high incorporation of EPA into plasma alkenylacyl GPE contributes to the enrichment of n-3 fatty acids into platelet phospholipids has yet to be determined, as

TABLE 3
Fatty Acid Composition in the 2-Position of Plasma Alkenylacyl GPE[a]

Fatty acid	Week 0	Week 3	Week 6	Week 9	Week 12
16:0	1.7 ± 0.9	1.3 ± 0.9	1.8 ± 0.8	2.1 ± 1.2	1.1 ± 0.6
18:0	0.8 ± 0.6	0.5 ± 0.3	1.0 ± 0.6	1.4 ± 1.0	0.6 ± 0.3
18:1n-9	5.0 ± 0.3[a]	2.9 ± 0.2[b]	3.5 ± 0.5[b]	4.9 ± 0.3a[a]	5.0 ± 0.5[a]
18:2n-6	13.9 ± 2.1[a]	5.2 ± 0.9b[b]	5.6 ± 1.1[b]	14.9 ± 1.9[a]	16.1 ± 2.1[a]
20:3n-6	2.7 ± 0.5[a]	0.6 ± 0.1[b]	0.7 ± 0.8[b]	2.8 ± 0.3[a]	2.6 ± 0.4[a]
20:4n-6	39.4 ± 4.5[a]	22.9 ± 2.9[b]	20.7 ± 2.4[b]	33.7 ± 3.3[a]	36.8 ± 4.1[a]
20:5n-3	5.0 ± 1.8[a]	28.2 ± 0.7[b]	29.6 ± 1.0[b]	6.4 ± 0.5[a]	3.7 ± 0.4[a]
22:4n-6	1.5 ± 0.2[a]	0.6 ± 0.1[b]	0.5 ± 0.0[b]	1.0 ± 0.1[a,b]	1.4 ± 0.1[a]
22:5n-3	4.7 ± 0.5	5.6 ± 0.5	5.2 ± 0.3	5.3 ± 0.2	4.8 ± 0.5
22:6n-3	19.6 ± 3.1[a]	29.6 ± 2.2[b]	27.6 ± 0.9[b,c]	23.0 ± 1.5[a,c]	19.5 ± 0.9[a]

*Subjects (n = 5) consumed MaxEPA for a 6-wk period followed by a 6-wk recovery period. At week 0 and after 3, 6, 9, and 12 wk, plasma alkenylacyl GPE was analyzed for fatty acid composition. Results are given in mol% as mean ± SEM. Values across each row having different superscripts are significantly different from each other ($P < 0.05$). Abbreviation as in Table 2.

this fraction represents a minor amount of the total EPA and DHA mass in plasma. Plasma/serum total GPE comprises only about 3% of the total plasma/serum phospholipids with the alkenylacyl class representing approximatley 60–70% of the total GPE (9–11). In contrast, GPC is the major phospholipid in plasma/serum (about 70% of total plasma/serum phospholipids) (9–11) and is therefore an abundant reservoir of EPA and DHA. The high incorporation of EPA and DHA into alkenylacyl GPE compared to diacyl GPE in plasma may be due to either a preferential synthesis/release of EPA/DHA-containing alkenylacyl versus diacyl GPE in/from liver and/or a slower clearance of n-3 containing alkenylacyl GPE from the circulation. Knapp *et al.* (18) have reported upon the selective incorporation of n-3 fatty acids in the inner membrane leaflet of alkenylacyl GPE of human erythrocytes upon supplementation with fish oil.

Although the hepatic concentration of alkenylacyl GPE is very low, the liver contains very active enzymes for the synthesis of this GPE class (19). Relative to hepatic concentrations of diacyl GPE, plasma alkenylacyl GPE levels are much greater (9–11), suggesting that the presence of active synthesizing enzymes for alkenylacyl GPE in liver may be coupled to efficient secretion of this phospholipid into plasma (19). Alkenylacyl GPE may also be cleared much more slowly from blood than the diacyl species, since a plasma phospholipase A_1 activity appears to be involved in clearing plasma phospholipids from the circulation (20,21). This enzyme cleaves the *sn*-1 position fatty acids from phospholipids, but does not act on the alkenyl group of GPE plasmalogen.

The relatively high level of alkenylacyl GPE in plasma relative to the diacyl species (9–11) leads to speculation on a potential function of GPE plasmalogen in plasma. One possible function may relate to an antioxidant role. The vinyl ether linkage of plasmalogens may protect cell membranes from oxidative damage by functioning as scavengers of reactive oxygen species (22,23). The presence of alkenylacyl GPE in plasma lipoproteins may serve to guard lipoproteins against oxidation. This may have implications in the development of atherosclerosis since oxidized, but not native, low density lipoprotein is rapidly taken up by macrophages. The lipid-laden foam cells characteristic of atherosclerosis are derived, at least in part, from macrophages (24). Interestingly, total plasma GPE was suggested to be an indicator in the prediction of atherosclerosis since elevated plasma GPE, more so than any other plasma lipid, was found to correlate with atherosclerotic complications (25). Distinction between diacyl versus alkenyl GPE and the constituent 2-positioned fatty acyl chains (n-6 vs. n-3) would be of interest in this regard.

In summary, we report herein on the enrichment of plasma plasmalogenic phospholipid with n-3 fatty acids following the human consumption of a fish oil concentrate. In particular, the marked enrichment of plasma alkenylacyl GPE with EPA, and the accompanying loss of AA, is of considerable interest. The potential importance of alkenylacyl GPE in plasma and the incorporation of n-3 fatty acids into this lipid has yet to be determined with respect to lipoprotein-mediated cardiovascular effects and modification thereof upon fish/fish oil consumption.

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Thioesterification of Platelet Proteins with Saturated and Polyunsaturated Fatty Acids

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ABSTRACT: We have demonstrated that more than 20 platelet proteins can be acylated with fatty acids via thioester linkages. These include the glycoprotein IX beta chain of glycoprotein Ib, components of the von Willebrand factor receptor on the platelet surface, P-selectin, and α subunits of G_z, G_q, and G_i. Our studies have shown that platelet proteins can be posttranslationally acylated in thioester linkages not only with palmitate but with myristate and also with the eicosanoid precursor fatty acids arachidonate and eicosapentaenoate. Thioesterification of platelet proteins with fatty acids other than palmitate may have significant functional consequences for reversible binding of proteins to membranes.
Lipids 31, S-217–S-221 (1996).

Acylation of proteins by fatty acids plays an important role in the interaction of proteins with hydrophobic membrane domains (1–5). Fatty acids can become covalently bound directly to proteins through amide linkages at *N*-terminal glycine residues and through thioester linkages to internal cysteines (1–3). Fatty acids can be indirectly yet covalently linked to proteins as part of the glycosyl-phosphatidylinositol (GPI) membrane anchor. In this complex molecule, fatty acids are part of the phosphatidylinositol moiety, and protein is linked to the carbohydrate-rich head group (4,5).

When fatty acids are incorporated into cells, the majority of them are esterified into phospholipids and triglycerides. A small amount, usually less than 2%, becomes acylated to protein (6–9). Acylation of proteins through an amide linkage occurs cotranslationally, and the fatty acid moiety does not turn over independent of the protein. The enzyme responsible for this type of modification, *N*-myristoyl transferase, has been purified, and gene coding for the protein has been cloned (10–12). This enzyme has been found to exhibit relatively rigid substrate specificity toward myristoyl-CoA as a fatty acid donor and the *N*-terminal glycine as fatty acid acceptor. A number of proteins of eukaryotic cells have been demonstrated to have amide-linked fatty acids. These include the insulin receptor α and β subunits (13), various G-protein α subunits (14–17), and the immunoglobulin heavy chain (18). Fatty acid acylation of proteins through thioester linkages occurs posttranslationally, and the fatty acid moiety attached to the protein turns over independent of the protein. Eukaryotic proteins with thioester-linked fatty acids include selected G-protein α subunits (14–17), the transferrin receptor (19), the sodium channel α subunit (20), rhodopsin (21), and ankyrin (22). As noted in this report, fatty acid acylation through thioester linkages has a much more relaxed fatty acid specificity than does fatty acid acylation through amide bonds (7,9). In the synthesis of GPI membrane-anchored proteins, addition of GPI to the protein occurs very early after synthesis of the protein. A preassembled GPI anchor is linked to the protein within the endoplasmic reticulum to produce a fully synthesized GPI-linked protein, which then makes its way to the outer leaflet of the plasma membrane. A number of cell surface proteins are bound to a GPI membrane anchor. These include alkaline phosphatase (23), decay accelerating factor (24), and the variant surface glycoprotein of trypanosomes (25).

Many studies have demonstrated significant functional consequences when a fatty acid is unable to covalently bind to protein when it is normally directed to do so. As three examples, p21Ras, a GDP/GTP–binding protein, can acquire the ability to transform cells if activated by mutations that favor the active GTP-bound form of the protein. Nonpalmitoylated mutants of activated p21Ras do not stably interact with membranes, remain predominantly cytosolic, and are unable to transform cells (26). G-protein α subunits associate with the cytoplasmic face of the plasma membrane where interactions with β-γ subunits, receptors, and effectors occur. Amino-terminal deletion mutants of α_q with removal of cysteines 9 and 10 cannot bind palmitate, are predominantly cytosolic, and are unable to activate phosphatidylinositol (PI)-specific phospholipase C (27). Finally, cysteine[341] in the carboxyl-terminal tail of the β_2 adrenergic receptor has been shown to be palmitoylated. Mutation of cysteine[341] to glycine results in a nonpalmitoylated form of this receptor and a markedly reduced ability to mediate isoproteronol stimulation of adenylylcyclase (28).

This report summarizes our studies identifying platelet proteins covalently modified by thioester-linked fatty acids and demonstrating relaxed specificity for fatty acids in

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Abbreviations: EPA, eicosapentacenoate; GPI, glycosyl-phosphatidylinositol; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis, PI, phosphatidylinositol.

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thioester linkage to proteins. Myristate, arachidonate, and eicosapentaenoate (EPA) were all found to become covalently bound to platelet proteins by thioester linkages.

EXPERIMENTAL PROCEDURES

Preparation of [^{3}H]palmitate-labeled platelet suspensions and periodate/sodium boro[^{3}H]hydride-labeled platelet suspensions, isolation of platelet glycoprotein Ib-IX complexes by immunoadsorption, preparation of platelet membrane fractions, lipid extraction of platelet proteins, release of fatty acids covalently bound to proteins and their identification by high-performance liquid chromatography (HPLC), and SDS–polyacrylamide gel electrophoresis (PAGE) and fluorography of polyacrylamide gels were all performed as previously described (6–9). Methods for preparation of [^{3}H]myristate-, [^{3}H]arachidonate-, and [^{3}H]eicosapentaenoate-labeled platelet suspensions have also been described in previous reports (7,9).

RESULTS

The platelet is an ideal system in which to study thioester linkage of fatty acids to proteins. Unlike the formation of covalent bonds between fatty acids and proteins through amide linkages or within GPI anchors, thioesterification of proteins with fatty acids is not dependent on protein synthesis. Since there is little if any protein synthesis in circulating platelets, protein acylation by fatty acids in platelets almost exclusively involves thioesterification. The first series of experiments demonstrates that

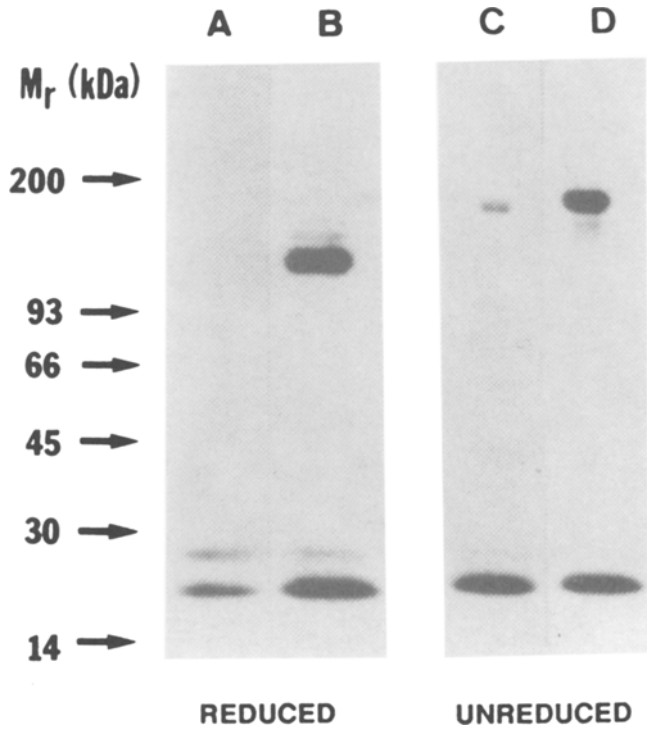


FIG. 2. Fluorogram of SZ.1-immunoprecipitated proteins from [^{3}H]palmitate-labeled platelets (lanes A and C) and from periodate/[^{3}H]$NaBH_4$-labeled platelets (lanes B and D). Platelets were isolated, labeled, and lysed, and immunoprecipitates were prepared as described in the Experimental Procedures section. Immunoprecipitated platelet proteins were solubilized in either reducing (lanes A and B) or nonreducing (lanes C and D) sample buffer and subjected to SDS–polyacrylamide gel electrophoresis on a 5–20% gradient gel followed by fluorography. Reprinted from *Journal of Biological Chemistry 264,* p. 9718, 1989.

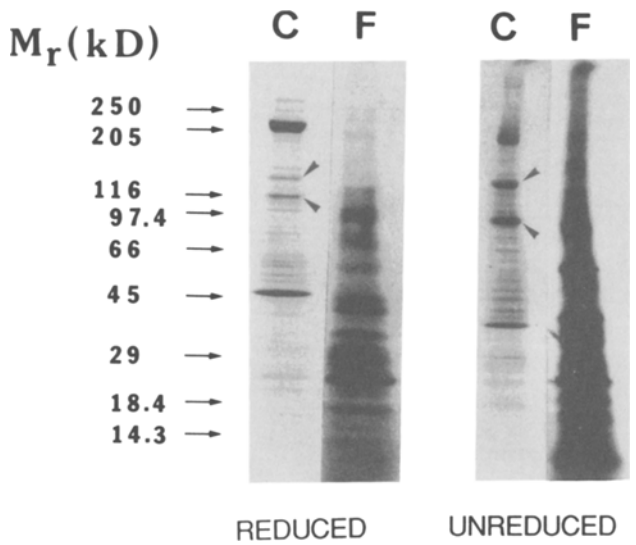


FIG.1. Recovery of [^{3}H]palmitoylated proteins in the membrane fractions of [^{3}H]palmitate-labeled platelets. The membrane fraction was isolated using a 1.0–3.5 mol/L continuous sorbitol gradient and solubilized in sample buffer with (reduced) or without (unreduced) β-mercaptoethanol and subjected to SDS–polyacrylamide gel electrophoresis. Gels were stained with Coomassie blue (C), and the radiolabeled polypeptides were visualized by fluorography (F). In each lane, 11 μg protein was applied to the gel. Glycoproteins IIb and IIIa are noted by arrowheads. Reprinted from *Blood 74,* p. 1345, 1989.

multiple platelet proteins can be esterified with radiolabeled palmitate through thioester linkages (6). Platelets were isolated by gel filtration or centrifugation and radiolabeled with 300 μCi/mL ^{3}H-palmitate for 45 min. Platelets were then delipidated extensively with multiple organic solvents. In some experiments, platelet proteins were evaluated by SDS–PAGE with protein stain or fluorography, and in other experiments platelets were processed for selective cleavage of fatty acids from proteins and HPLC of liberated fatty acids for fatty acid identification. In these experiments, approximately 1–2% of the palmitate incorporated into cells became covalently bound to protein. As shown in Figure 1, in lanes marked C for Coomassie stain, several major and many minor platelet membrane proteins are visible. Lanes marked F for fluorography in Figure 1 indicate proteins that became palmitoylated. It should be noted that there is no correspondence between the pattern of prominent proteins in the Coomassie-stained lanes and that of the intensely labeled proteins in the fluorography lanes. Extensive removal of noncovalently bound lipids by extraction with a series of solvents produced no changes in the fluorographic pattern. This indicates that fatty acids were not bound to proteins by nonspecific trapping. Following labeling with [^{3}H]palmitate,

platelet proteins were treated with alkaline methanol, which selectively releases fatty acids in ester linkage but does not release fatty acids in amide linkage. Methyl palmitate was recovered almost exclusively, indicating that the radioactivity in the SDS gels represented fatty acids, and not nonfatty acid metabolites. In support of this finding, hydroxylamine at neutral pH, which liberates thioester-linked fatty acids but not amide- or 0-ester–bound fatty acids, released radiolabeled palmitate from proteins to which it was bound.

In other studies we demonstrated that platelet glycoproteins Ib and IX, components of the platelet von Willebrand factor receptor, are palmitoylated. Platelets were isolated and then radiolabeled with [^{3}H]palmitate or sodium periodate/sodium boro [^{3}H]hydride. The platelets were lysed, and immunoprecipitation of the glycoprotein Ib-IX complex was performed using the SZ.1 monoclonal antibody (8). SDS–PAGE and fluorography were then performed to determine if the glycoprotein Ib α or β chain or glycoprotein IX was acylated with fatty acid. In Figure 2, in lanes A and C, proteins labeled with palmitate are visible. In lanes B and D, glycoproteins Ib and IX labeled with sodium periodate/sodium borohydride are shown as standards. SDS gel electrophoresis and fluorography of immunoprecipitates from [^{3}H]palmitate-labeled platelets revealed two radiolabeled bands under reducing conditions at 24 kDa and 19 kDa and 2 bands under nonreducing conditions at 170 kDa and 19 kDa. As demonstrated by comparison to the immunoprecipitates from sodium periodate/sodium boro[^{3}H]hydride-labeled platelets, [^{3}H]palmitate-labeled bands under reducing conditions correspond to glycoproteins Ib β and IX. [^{3}H]Palmitate-labeled bands under nonreducing conditions correspond to glycoprotein IX and intact glycoprotein Ib. In separate experiments, the immunoprecipitate was delipidated, and the remaining covalently bound fatty acid residues were selectively cleaved from the protein and analyzed by HPLC to identify thioester-linked fatty acids. Alkaline methanolysis followed by HPLC demonstrated that the radioactivity associated with the glycoprotein Ib-IX complex from the [^{3}H]palmitate-labeled platelets was covalently bound [^{3}H]palmitate in ester linkage to protein. Release of [^{3}H]palmitate covalently bound to glycoproteins Ib β and IX by neutral hydroxylamine further indicated that the protein and fatty acid were linked by way of a thioester bond. In other studies, we demonstrated that the platelet protein P-selectin (29) can be acylated with palmitate and stearate through thioester linkages. We also showed that palmitate and arachidonate can become bound in thioester linkage to α subunits of G_z, G_q, and G_i (30).

In more recent studies, we demonstrated that myristate can become covalently bound to platelet proteins *via* thioester linkages (7). This finding was unexpected, since myristate is known to be the predominant amide-linked fatty acid. We found that myristate was releasable with alkaline methanol from platelet proteins prelabeled with [^{3}H]myristate, which, as noted earlier, releases ester-linked but not amide-linked fatty acids. In these experiments, we also found that palmitate competed with myristate for protein binding, that myristate and palmitate bound to the same platelet proteins, and that myristate was released from protein by neutral hydroxylamine. As shown in Figure 3, the bands in the lanes marked

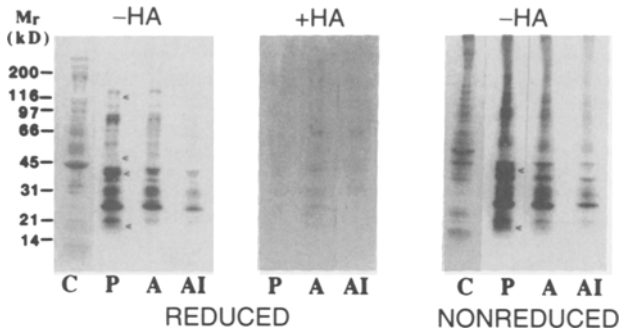


FIG. 4. [^{3}H]arachidonoylated and [^{3}H]palmitoylated proteins in platelets. Washed platelets were labeled with [^{3}H]fatty acid. Platelet proteins were then precipitated, extracted, and dissolved in sample buffer. Reduced and nonreduced samples (40 μg protein/well) were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE), after which the radiolabeled proteins were detected by fluorography. SDS–PAGE of reduced samples was performed in duplicate on two parallel gels, and one of the fixed gels was subjected to neutral hydroxylamine (+HA) treatment. Lanes C, Coomassie-stained gels of platelet proteins; lanes P, [^{3}H]palmitate-labeled platelets; lanes A, platelets incubated with [^{3}H]arachidonate; lanes AI, [^{3}H]arachidonate-labeled platelets incubated with baicalein and indomethacin to inhibit cyclooxygenase and lipoxygenase. Horizontal lines on the left side of first gel represent migration distance of M_r marker proteins. Arrowheads on the P lane identify bands that can be detected only on the fluorogram of ^{3}H-palmitoylated proteins. Reprinted from *Journal of Biological Chemistry 268,* p. 18246, 1993.

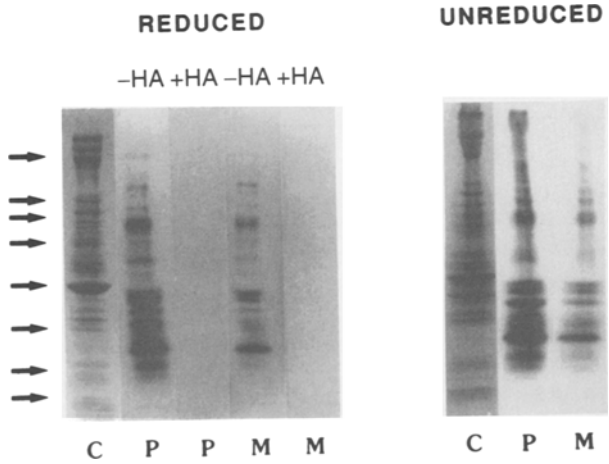


FIG. 3. [^{3}H]myristoylated and [^{3}H]palmitoylated proteins in platelets. Platelets were washed and labeled with [^{3}H]fatty acid; platelet proteins were precipitated and extracted with acetone, then analyzed with SDS–polyacrylamide gel electrophoresis and fluorography as described in the Experimental Procedures section. C, Coomassie blue staining; M and P, fluorogram of proteins from [^{3}H]myristate- (4-week exposure) and [^{3}H]palmitate- (4-day exposure) labeled platelets, respectively; –HA and +HA, gels incubated in the absence or presence of 1 M neutral hydroxylamine. To each well, 63 μg platelet protein was applied. Arrows represent migration distance of standard proteins of the following molecular mass, from top to bottom: 200, 116, 97.4, 66.2, 45, 31, 21.5, and 14.4 kDa. Reprinted from *Journal of Biological Chemistry 268,* p. 8255, 1993.

P for [^{3}H]palmitate label and M for [^{3}H]myristate label are essentially identical, indicating that myristate and palmitate bind to the same platelet proteins. As noted by the lanes marked +HA for hydroxylamine treatment, myristate has a sensitivity equivalent to palmitate for release from proteins.

We subsequently tested whether arachidonate and eicosapentaenoate can become covalently bound to proteins *via* thioester linkages (9). In these experiments, radiolabeled arachidonate or EPA was added to platelets, followed by SDS–PAGE and fluorography. In some experiments, cyclooxygenase and lipoxygenase inhibitors were added to assess the possibility that the arachidonate and EPA were first being converted to oxygenated metabolites and then becoming covalently bound to proteins. As shown in Figure 4, arachidonate and palmitate bound to most of the same proteins. There were a few proteins, noted by the arrows, that were palmitoylated and apparently not arachidonoylated. We were also able to demonstrate that arachidonate was released from protein by neutral hydroxylamine. The lanes marked +HA in Figure 4, which are markedly overexposed to permit visualization of the faint bands present, illustrate that neutral hydroxylamine was able to release the arachidonate covalently bound to proteins. Protein-bound arachidonate was slightly less sensitive to neutral hydroxylamine for release than was protein-bound palmitate. Figure 4 also shows that, in the presence of cyclooxygenase and lipoxygenase inhibitors (in lanes marked AI), there was essentially no difference in fatty acid binding to protein, although the lanes representing samples with inhibitors were somewhat less intense than those without inhibitors. The basis for this difference remains unexplained. This result indicates that the fatty acids, and not their oxygenated metabolites, bound to proteins. In other studies, we demonstrated that arachidonate and EPA were released from platelet proteins by alkaline methanol, further supporting the conclusion that these fatty acids, like myristate and palmitate, can become bound to proteins through thioester linkages. In Figure 5, it can be seen that arachidonate and EPA bind to the same platelet proteins. The more intense labeling of platelet proteins by arachidonate may reflect the slightly higher concentration of radiolabeled arachidonate (4 μM) relative to EPA (2.6 μM) used in these experiments.

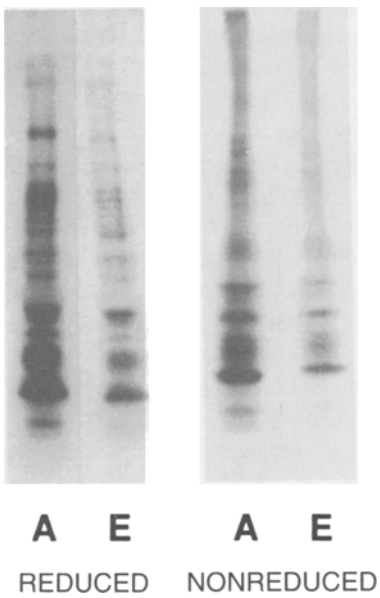


FIG. 5. Fluorograms of platelet proteins labeled with [^{3}H]arachidonate and [^{3}H]EPA. Reduced and unreduced protein samples (50 μg protein/well) of platelets labeled with [^{3}H]arachidonate (A) and [^{3}H]eicosapentaenoic acid (E) were analyzed by SDS–polyacrylamide gel electrophoresis and fluorography. Reprinted from *Journal of Biological Chemistry 268,* p. 18247, 1993. Abbreviations as in Figure 4.

DISCUSSION

Our studies indicate that many platelet proteins are thioesterified with fatty acids, including glycoprotein Ib β (8), glycoprotein IX (8), P-selectin (29), and α subunits of G_z, G_q, and G_i (30). In addition, we demonstrated that the fatty acid capable of binding to proteins in thioester linkage is not only palmitate. Myristate (7); the eicosanoid precursor fatty acids, arachidonate and eicosapentaenoate (9); and, from our studies with P-selectin (29), stearate can all become protein-bound in a thioester linkage. Posttranslational binding of myristate, palmitate, arachidonate, and EPA to platelet proteins apparently involves identical mechanisms, as these fatty acids become bound to the same set of substrate proteins. The binding of myristate or stearate instead of palmitate to proteins *via* thioester linkages may have significant consequences for membrane binding. Unlike palmitoylated proteins, proteins that are covalently bound to myristate can be found in the cytoplasm (31). Myristate is two carbons shorter than palmitate and therefore somewhat less efficient in promoting membrane binding than is palmitate. Stearate is two carbons longer than palmitate and may be more efficient than palmitate in promoting membrane binding. We speculate that there are significant functional consequences to protein myristoylation or stearoylation at internal cysteines rather than palmitoylation. On a structural basis, thioesterification with arachidonate or EPA may have even greater functional consequences for membrane binding. Myristate, palmitate, and stearate are all saturated fatty acids that have no bends in the fatty acid molecule. Arachidonate with 4 double bonds and EPA with 5 are highly twisted fatty acid molecules. Studies comparing the relative affinity for membranes of arachidonoylated proteins vs. proteins bound to saturated fatty acids have not yet been performed. Our studies with G-proteins show that several G-protein α subunits can be thioesterified with arachidonate as well as with palmitate (30).

There is another significant issue related to arachidonoylation of proteins. Upon cell activation, significant amounts of arachidonate are liberated from phospholipids by phospholipase action, and a large amount of free arachidonate is present in the cytoplasm of the cell for a brief time (32). This arachidonate is potentially available for thioesterification of proteins *via* displacement of saturated fatty acids or binding to

sites on the protein where saturated fatty acids are not present. Modification of proteins with arachidonate in the cell activation process may be an important component of protein regulation in transmission of intracellular signals. Determination of the actual amounts of palmitate, myristate, stearate, arachidonate, and EPA that are bound to platelet proteins by thioester linkages *in vivo* will be established by studies in which the thioester-linked fatty acids in the native state are specifically removed and analyzed quantitatively without the use of radiolabeled fatty acids. These studies are currently in progress in our laboratory.

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Membrane-Associated and Cytoplasmic Fatty Acid-Binding Proteins

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ABSTRACT: A number of cellular fatty acid-binding proteins are being implicated in the uptake and intracellular transport of long-chain fatty acids by parenchymal cells. Having been a topic of research for more than 20 years, cytoplasmic fatty acid-binding proteins now are assigned various pivotal functions in intracellular fatty acid transport and metabolism. More recently several membrane-associated fatty acid-binding proteins have been identified and these proteins are thought to function in the transmembrane transport of fatty acids. In this review, a short summary is provided of the latest developments in this research area.

Lipids 31, S-223–S-227 (1996).

Long-chain fatty acids (FA) are important compounds serving as substrates for energy-production and formation of phospholipids (1). Moreover, they can participate in signal transduction pathways (2–4). Adipose tissue takes up large amounts of FA from blood plasma for storage as intracellular triacylglycerols. In contrast, oxidative skeletal muscles and especially the heart take up FA as fuel for contractile processes. Since FA are poorly soluble in water the rapid metabolism of these FA would probably not be possible without special binding proteins, which dramatically increase the solubility of these compounds in aqueous environments. In blood and interstitium, FA are mainly bound to albumin, keeping the unbound FA concentrations extremely low (<10 nM) (5). Intracellularly, FA are most likely bound to specific cytoplasmic fatty acid-binding proteins (FABP) (6–10). More recently, membrane-associated proteins have been hypothesized to function in the cellular uptake of FA. These membrane and cytoplasmic proteins will be discussed in more detail below.

Membrane-associated FABP. The mechanism of transmembrane transport of FA into parenchymal cells has been a matter of debate for several years. Some investigators favor a mechanism in which FA cross the membrane by simple diffusion (11,12). Others found saturable uptake of FA in several cell-types, a process that could be inhibited by FA analogues, indicating that membrane-associated proteins are involved (13). Subsequently, by using various techniques, a number of such membrane proteins have been identified (Table 1). The first membrane protein described to be involved in the uptake of FA is a 40 kDa protein present in the plasma membrane of rat liver cells (14). This protein, $FABP_{PM}$, was subsequently also found in intestine (15), and heart (16). The protein seems closely related, if not identical, to mitochondrial aspartate aminotransferase (17). $FABP_{PM}$ is not an integral membrane protein but has been shown to possess affinity for membranes (17).

In 1987, Fujii *et al.* (18,19) described another membrane protein with high affinity for FA, which was designated fatty acid receptor (FAR). This protein of about 60 kDa was found to be present in kidney and heart. The third membrane protein putatively involved in FA uptake, identified in adipocytes by Harmon and co-workers (20) and recently cloned by Abumrad *et al.* (21), is an 88 kDa protein called fatty acid translocase (FAT). FAT from rat was found to be highly homologous (85%) to the human leucocyte differentiation antigen CD36, a receptor protein present among others on monocytes and platelets, and thought to be involved in adhesion phenomena and intracellular signalling (for review on CD36 see ref. 22). Comparison of rat FAT (21) and the recently cloned mouse CD36 (23) revealed an amino acid identity of 93%, which strongly suggests that these proteins are species homologues. Recent investigations showed that CD36 is an integral membrane protein with one transmembrane region (24).

In adipocytes, Trigatti *et al.* (25) found another membrane protein implicated in the transmembrane transport of FA. This 22 kDa protein was identified by photoaffinity labelling with a FA analogue. The final membrane protein hypothesized to augment cellular FA uptake that has been found up till now, is called fatty acid transport protein (FATP) (26). A functional approach using expression cloning was followed to identify this 63 kDa protein in adipocytes. FATP was shown to be an integral membrane protein present in the plasma membrane of several tissues, and has been predicted to have several membrane spanning domains (26).

The precise functions of the FA-binding membrane-associated proteins in the transport of FA across cellular membranes are still unclear. Some of the possible mechanisms by which membrane proteins could be involved in this process

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Abbreviations: FA, long-chain fatty acids; FABP, fatty acid-binding protein; FAR, fatty acid receptor; FAT, fatty acid translocase; FATP, fatty acid transport protein.

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TABLE 1
Membrane-Associated Fatty Acid-Binding Proteins (FABP)

Protein	Molecular mass (kDa)	Major occurrence	Reference
Membrane FABP	22	Adipose tissue	25
$FABP_{PM}$	40	Liver, heart, adipose tissue, intestine	15,16
FA receptor	56–60	Heart, kidney	18,19
FA transport protein	63	Adipose tissue, heart, skeletal muscle	26
FA translocase	88	Adipose tissue, heart, skeletal muscle	20,21

are shown in Figure 1. These proteins might function as a translocator, but it is also possible that they represent acceptors for FA released from albumin and that FA subsequently cross the plasma membrane by diffusion through the phospholipid bilayer. Albumin binding proteins have been described which could play a role in this mechanism (27). Furthermore, there might be a direct interaction between a membrane-associated FA-binding protein and (extracellular) albumin and/or (intracellular) FABP. Such interactions would ensure a controlled uptake mechanism in which FA remain protein-bound. Further characterization of the FA-binding membrane proteins will provide a better understanding of the complex transmembrane transport mechanisms of FA.

Intracellular FABP. Cytoplasmic FABP belong to a single gene family of intracellular lipid binding proteins of 14–15 kDa, capable of binding hydrophobic ligands with high affinity (28). The mammalian cytoplasmic lipid-binding proteins are listed in Table 2. Since the discovery of FABP in 1972 by Ockner and co-workers (29), nine different FABP, including the recently discovered testicular 15 kDa protein (T-LBP) (30,31), have been identified (Table 2). All lipid binding proteins are composed of two α-helices and 10 anti-parallel β-strands, organized in two β-sheets, thereby forming a clam shell-like structure (8). The lipid ligand is bound inside the molecule by interaction with specific amino acid residues within the binding pocket of the lipid binding protein (8). A recent investigation by Richieri *et al.* (32) suggests that the FABP have a higher affinity for the binding of FA than found in previous studies (7,9,10) with the dissociation constants (Kd) ranging from 2 to 1000 nM, depending on type of FABP and FA (32). A striking feature of the FABP is their relative abundance in tissues with active FA metabolism.

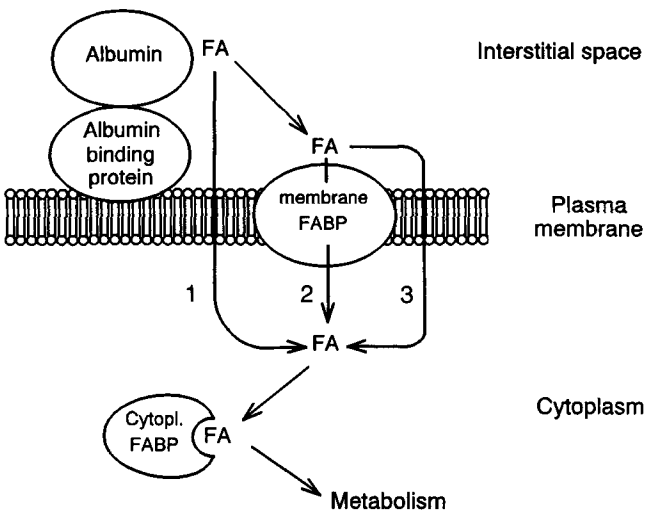


FIG. 1. Schematic presentation of the mechanism of cellular fatty acid uptake and the possible role of membrane-associated fatty acid-binding proteins in this process. Transmembrane transport could take place without the involvement of membrane proteins (1), or with a membrane protein acting as a true translocator (2), or as an acceptor for fatty acids thus, creating a steeper transmembrane gradient (3). FA, long-chain fatty acid; membrane FABP, any membrane-associated fatty acid-binding protein (see Table 1); cytopl. FABP, any cytoplasmic FABP (see Table 2).

A number of biological roles have been ascribed to the FABP: (i) Facilitation of the transport of FA to their intracellular sites of utilization. Indirect evidence was provided in studies showing a correlation between FA-utilization and FABP-content (33,34) and by showing that *in vivo* FA are bound to FABP (35,36). Furthermore, *in vitro* studies showed an increase in FA transport from isolated mitochondria to artificial phospholipid vesicles in the presence of H-, or L-FABP (37). Theoretical studies also support a role for FABP in FA-transport in cardiomyocytes (38). More direct evidence was found by transfection studies in which cells transfected with H-, L-, or A-FABP showed increased FA-uptake rates (39-41). Finally, a recent report showed that a single amino acid substitution (Ala54 -> Thr54) in human I-FABP, as occurs in some Pima Indians, causes a two-fold greater affinity for FA. This was associated with increased fat oxidation rates and with insulin resistance, also suggesting a role for I-FABP in cellular FA-transport (42). (ii) Prevention of local high FA concentrations and thereby protecting the cell against detrimental effects of FA, for instance in the ischemic heart (43). (iii) By modulating hydrophobic ligand metabolism, FABP can influence important cellular events like mitogenesis (44) and FA-mediated signal transduction pathways (3,7). Recently, a small fraction of H-FABP in rat heart and mammary gland was found to be phosphorylated upon stimulation with insulin, and it was suggested that H-FABP might play a role in signal transduction downstream from the insulin receptor (45,46). (iv) Inhibition of growth and induction of differentiation of cultured mammary epithelial cells has been reported as an extracellular function of mammary derived growth in-

TABLE 2
Mammalian Cytoplasmic Lipid-Binding Proteins[a]

Protein	Current designation	Other designations	Ligand	Major occurrence
Heart FABP	H-FABP	MDGI	FA	Heart, skeletal muscle, smooth muscle, brain, kidney, mammary gland
Liver FABP	L-FABP	Z-protein	FA, heme, bilirubin, prostaglandins	Liver, small intestine, kidney
Intestinal FABP	I-FABP		FA	Small intestine
Adipocyte lipid-binding protein	A-LBP	A-FABP, aP2	FA, retinoic acid	Adipose tissue
Epidermal FABP	E-FABP		FA	Epidermis
Brain FABP	B-FABP		FA	Nervous system
Myelin FABP	M-FABP	Myelin-P2	FA, retinoids	Nervous system
Ileal lipid-binding protein	I-LBP	I-BABP, gastrotropin	FA, bile acids	Small intestine (ileum)
Testicular lipid-binding protein	T-LBP	PERF15	ND	Testis
Cellular retinoic acid-binding protein	cRABP		Retinoic acid	Testis, nervous system, kidney, skin
Cellular retinoic acid-binding protein II	cRABPII		Retinoic acid	Skin, adrenals
Cellular retinol-binding protein	cRBP		Retinol	Widespread expression
Cellular retinol-binding protein II	cRBPII		Retinol	Intestine

[a]Data are compiled from References 3, 8, 30, 31, and 56. ND, not determined; FA, long-chain fatty acids, other abbreviation as in Table 1.

hibitor, which is identical to H-FABP (9). (v) Induction of myocyte hypertrophy through binding with a high affinity receptor is another extracellular function recently ascribed to H-FABP (47).

Regulation of expression of FABP. The expression of A-FABP and also of FAT and FATP is upregulated during differentiation from preadipocytes to adipocytes (21,26,48), and is paralleled or preceded by an increased uptake of FA (48). Recently, it was shown that FA themselves can induce the expression of A-FABP in rat preadipocytes (49).

In cardiac cells, H-FABP expression increases markedly during the first few days after birth and in the weaning period (50,51). During this period, the FA oxidation capacity is also increased (52). Recently, it was shown that FAT is co-expressed with H-FABP in rat muscles and that FAT expression followed a similar upregulation in heart during development, indicating related biological functions (53).

Diabetes is a disease in which several organs, like the heart, rely more on FA oxidation as their major energy source. In streptozotocin-induced diabetic animals, a decrease of A-FABP expression (54) and an increase in H-FABP expression (55) have been shown. These results indicate that insulin deficiency can influence FABP expression levels. Preliminary results from our laboratory show that FAT is also upregulated in rat muscles in streptozotocin-induced diabetic rats (Fig. 2). These results suggest that altered FA-handling of cells can influence FABP and FAT expression.

Concluding remarks. In the last 20 years, a number of cellular proteins with affinity for FA have been identified. Among these are nine different types of intracellular 15 kDa FABP which most likely function in facilitating the *trans*-cytoplasmic transport of FA. In the last few years, more interest in the transmembrane transport of FA has resulted in the discovery of membrane proteins that might be involved in this process. Now that the genes of most of these proteins have been cloned, molecular biological techniques can be applied to manipulate the cellular content and binding activity of

FIG. 2. Northern blot analysis of total RNA from liver, heart, soleus, and extensor digitorum longus (EDL) muscle from control and streptozotocin-induced diabetic rats using fatty acid translocase cDNA as probe.

these proteins. This might provide a better understanding of their role in the complex mechanisms by which FA are taken up and distributed in the cell.

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Specificity of Polyunsaturated Fatty Acid Release from Rat Brain Synaptosomes

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ABSTRACT: Release of specific polyunsaturated fatty acids from cell membranes may have a significant implication in biological function, considering the involvement of various fatty acids in cell signal transduction. In the present study, release of polyunsaturated fatty acids from rat brain synaptosomes by endogenous synaptosomal lipase activity was examined in comparison to that by cobra venom phospholipase A_2 (Naja naja naja). Cobra venom phospholipase A_2 (Naja naja naja) preferentially hydrolyzed docosahexaenoic acid (22:6n-3) from both synaptosomes and lipid mixtures containing similar classes of lipids commonly found in the brain. Arachidonic acid (20:4n-6) and oleic acid (18:1n-9) were also hydrolyzed; however, monoene species was hydrolyzed slower than were polyenoic species in synaptosomes. Phosphatidylethanolamine was the most preferred phospholipid class for release of 22:6n-3 fatty acid from both lipid mixtures and synaptosomes.

In contrast to hydrolysis by cobra venom phospholipase A_2, endogenous synaptosomal lipase activity preferentially hydrolyzed 20:4n-6 from rat brain synaptosomes, despite the high abundance of 22:6n-3 in synaptosomal membranes. Preferential release of 20:4n-6 was observed over a wide range of pH values and calcium concentrations. Synaptosomal 22:6 species appeared to be resistant to hydrolysis even after stimulation with various agents such as phorbolmyristate, suggesting that physiological importance of 22:6n-3 in neuronal membranes may not be as the release fatty acid.
Lipids 31, S-229–S-233 (1996).

Phospholipase A_2 (PLA_2) hydrolyzes the *sn*-2 fatty acyl chain from phospholipids producing free fatty acids and lysophospholipids (1,2). Hydrolysis of polyunsaturated fatty acids from their reservoir phospholipids occurs primarily by the PLA_2 reaction, since most of the polyunsaturated fatty acids are esterified at the *sn*-2 position of the phospholipids. Released polyunsaturated fatty acids can be metabolized to many potent biological mediators such as prostaglandins and leukotrienes (3). Lysophospholipids resulting from the PLA_2 reaction are strong surfactants and can modify physical and chemical characteristics of cell membranes significantly. Therefore, activation of PLA_2 can bring about many pathophysiological consequences, especially in cells where polyunsaturates are highly concentrated.

In neuronal membranes, docosahexaenoic acid (22:6n-3) is the most abundant polyunsaturated fatty acid esterified in phospholipids (4,5). Although 22:6n-3 is thought to be essential for proper neuronal and retinal functions (4,5), mechanisms involved in its essentiality are not clearly understood. It is possible, however, that selective release of 22:6n-3 may play an important role in cell signaling, as has been suggested for arachidonic acid (20:4n-6) selectively hydrolyzed by cytosolic PLA_2 (6,7). To test this possibility, we examined specificity in the release of polyunsaturated fatty acids from brain synaptosomes where 22:6n-3 is especially enriched, using a well-characterized cobra venom PLA_2 (Naja naja naja). The observed substrate specificity was compared with results obtained from lipid mixtures containing various classes of lipids commonly found in the brain. Specificity of release of 20:4n-6 and 22:6n-3 by endogenous synaptosomal lipase activity was characterized subsequently.

MATERIALS AND METHODS

All phospholipid standards were either purchased from or custom-synthesized by Avanti Polar Co. (Alabaster, AL). All inhibitors and stimulators used in this study were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of lipid mixture. Lipid mixtures were prepared according to Bayburt et al. (8), with slight modifications. To minimize alterations in characteristics of lipid mixtures, 85% of the lipid composition was kept constant and only 15% of the

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Abbreviations: DG, diacylglycerol; di-16:0-, di-palmitoyl-; 18:0/18:1-,1-stearoyl-2-oleoyl-; 18:0 20:4-, 1-stearoyl-2-arachidonoyl-; 18:0 22:6-, 1-stearoyl-2-docosahexanoyl-; 18:1n-9, oleic acid; 18:2n-6, linoleic acid; Gro*P*Cho, glycerophosphocholine; Gro*P*Etn, glycerophosphoethanolamine; Gro*P*Ino, glycerophosphoinositol; Gro*P*OH, glycerophosphoric acid; Gro*P*Ser, glycerophosphoserine; HPLC, high-performance liquid chromatography; NANA, N-acetylneuraminic acid; NGN, N-glycolylneuraminic acid; PLA_2, phospholipase A_2; PLC, phospholipase C; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine; PtdSer/PtdOH, mixture of PtdSer and PtdOH in the ratio of 1:1 determined by HPLC.

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lipids was altered with phosphatidylcholine (PtdCho), phosphatidylethyanolamine (PtdEtn), or phosphatidylserine/phosphatidic acid (PtdSer/PtdOH) species. Total 15 μmoles of lipids [PtdCho, 20%; PtdEtn, 25%; cholesterol, 40%; phospholipid mixture containing equimoles of 1-stearoyl-2-docosahexanoyl- (18:0 22:6-), 1-stearoyl-2-arachidonoyl- (18:0 20:4-), and 1-stearoyl-2-oleoyl- (18:0 18:1-) glycerophosphocholine (Gro*P*Cho), glycerophosphoethanolamine (Gro*P*Etn), or glycerophosphoserine/glycerophosphoric acid (Gro*P*Ser/Gro*P*OH) 15%] were sonicated in 1 mL deionized water for 15 s under nitrogen. This composition was based on the reported composition of PtdCho, PtdEtn, and cholesterol in rat brain corresponding to 20, 23.6, and 39.3%, respectively (9). The suspension was frozen on dry ice and then sonicated in water for 30 min before dilution. Each incubation mixture contained 1.5 μmoles total lipids in 1 mL PLA_2 buffer (25 mM *tris*-HCl, 0.1 mM KCl, and 10 mM $CaCl_2$, pH 8.0). Alternatively, lipid mixtures were prepared by sonicating dried mixed lipids in the PLA_2 buffer for 30 min.

Preparation of synaptosomes. Synaptosomes were prepared according to Cotman (10). Synaptosomes were washed to remove Ficoll reagent by resuspending in excess phosphate-buffered saline buffer (pH 7.4) and centrifuging at 9,000 × g for 10 min at 4°C. The washed synaptosomes were reconstituted in chilled PLA_2 buffer devoid of $CaCl_2$ to a concentration of 0.167 mg protein/mL solution. Synaptosomes were incubated for 0–60 min at 37°C in the presence of $CaCl_2$ (final concentration of 10^{-7}–10^{-3} M) and 5 mg/mL fatty acid free-bovine serum albumin. Each incubation mixture contained 0.75 mL (0.125 mg protein) synaptosome suspension.

Incubation and lipid analysis. Lipid mixtures or synaptosomes were incubated with or without 0.1 unit cobra venom PLA_2 (Naja naja naja, 1,800 unit/mg protein, Sigma, Chemical Co.) at 37.5°C for 0–60 min. Appropriate inhibitors and stimulators were coincubated with synaptosomes. The reaction was terminated by acidification to pH 3.0 with formic acid, and the incubation mixture was extracted according to Bligh and Dyer (11) in the presence of 23:0 as an internal standard. An aliquot was transmethylated for gas chromatographic determination of the individual fatty acid content in the total lipid extract. Separation of various lipid classes was achieved using aminopropyl solid phase extraction columns (Varian, Harbor City, CA), as described previously (12). Separated lipids were transmethylated with BF_3-methanol (14% wt/vol, Alltech, Deerfield, IL) for 2 h at 100°C under nitrogen, according to Morrison and Smith (13), and subjected to gas chromatographic analysis, as described previously (12). Alternatively, extracts of the incubation mixture were subjected to thermospray liquid chromatography–mass spectrometry analysis, as has been described (14).

RESULTS

Studies using lipid mixtures. When lipid mixtures were incubated with 0.1 unit cobra venom PLA_2, a steady but nonlinear increase of free fatty acids was observed (Fig. 1). After the extent of hydrolysis exceeded 10% of the substrate, only a slight progress in hydrolysis was observed, as in the case with the lipid mixture containing the PtdEtn mixture (Fig. 1B). Cleavage of 22:6n3 fatty acid, however, was consistently the highest for each phospholipid class at any incubation time-point examined. Lipid mixtures obtained by both methods described in the experimental section generated qualitatively similar results, except that lipid mixtures generated by suspending lipid mixtures directly in the PLA_2 buffer by sonication yielded hydrolysis products at a slightly higher level. Hydrolysis of 20:4n-6 and oleic acid (18:1n-9) was comparable, although a slight preference of 20:4n-6 over 18:1n-9 was observed for lipid mixtures containing PtdEtn mixtures. Among phospholipid classes examined, PtdEtn was hydrolyzed most quickly, while PtdCho hydrolysis was least favored.

Hydrolysis of rat brain synaptosomes by cobra venom PLA_2. Figure 2 shows the time course of PLA_2 hydrolysis of 18:1, 20:4, and 22:6 containing phospholipids in synaptosomes. As was the case with the lipid mixtures, 22:6n-3 was the fatty acid found at the highest level in the incubation mixture at any incubation time-point. Cleavage of 20:4n-6 and 18:1n-9 species were consistently lower than for 22:6n-3 species. Synaptosomes contain 12–13% of 20:4n-6 and 19–20% of 22:6n-3 in total lipids. Although the fatty acyl content of 18:1n-9 and 20:4n-6 in synaptosomes was comparable, PLA_2 appeared to hydrolyze 20:4n-6 more favorably than 18:1n-9. When the extent of hydrolysis was expressed as the percentage of the cleaved fatty acid against the level of the individual fatty acid present in the total lipids (Fig. 2B), qualitatively similar results were obtained, indicating that the high level of cleaved 22:6n-3 is not just due to the high abundance of the substrate in synaptosomes.

To find the origin of the cleaved fatty acids, PLA_2 hydrolysis was examined at the molecular species level, using thermospray liquid chromatography–mass spectrometry (15,16). The diacylglycerol fragments of the major phospholipid molecular species were monitored selectively, as shown in Fig. 3, since they were the most abundant peak in the thermospray spectra and contained the molecular species information (15,16). For free fatty acids, $(M + NH_4)^+$ ions were selectively monitored. Since diacylglycerol fragments were common for all glycerophospholipid classes containing a given fatty acyl composition, monitoring these ions in conjunction with on-line high-performance liquid chromatography (HPLC) separation facilitated analysis of molecular species in complex mixtures. Although the intensity of these ions did not directly represent the absolute level of the respective species (14,17), comparison of chromatographic peak areas of individual molecular species before and after PLA_2 hydrolysis allowed us to monitor hydrolysis at the molecular species level. After 1 min incubation, 22:6n-3 and 20:4n-6 appeared in the chromatogram with the former at a higher level. Decrease of these species was observed from 18:0 22:6-Gro*P*Etn, 18:0 20:4-Gro*P*Ino, and 18:0 20:4-Gro*P*Etn, indicating that 22:6n-3 and 20:4n-6 were hydrolyzed initially from these species. Hydrolysis of 18:0 22:6-Gro*P*Ser was also indicated but at a lower level in comparison to 18:0 22:6-Gro*P*Etn. Further incubation decreased

18:0 22:6 and 18:0 20:4 species of all classes, as well as 18:0 18:1 and 16:0 18:1-Gro*P*Cho significantly. Decrease of 16:0 20:4-Gro*P*Cho was also observed, but to a much lesser extent. In fact, 16:0 20:4-Gro*P*Cho became the dominating 20:4 containing species after 30 min of incubation (data not shown). It also appeared that 18:0 18:1-Gro*P*Cho was more favorably hydrolyzed than was 16:0 18:1-Gro*P*Cho. Analysis of individual lipid classes by gas chromatography after separation by solid phase extraction (12) indicated similar results.

Hydrolysis of rat brain synaptosomes by endogenous lipase activity. Endogenous lipase activity was also observed

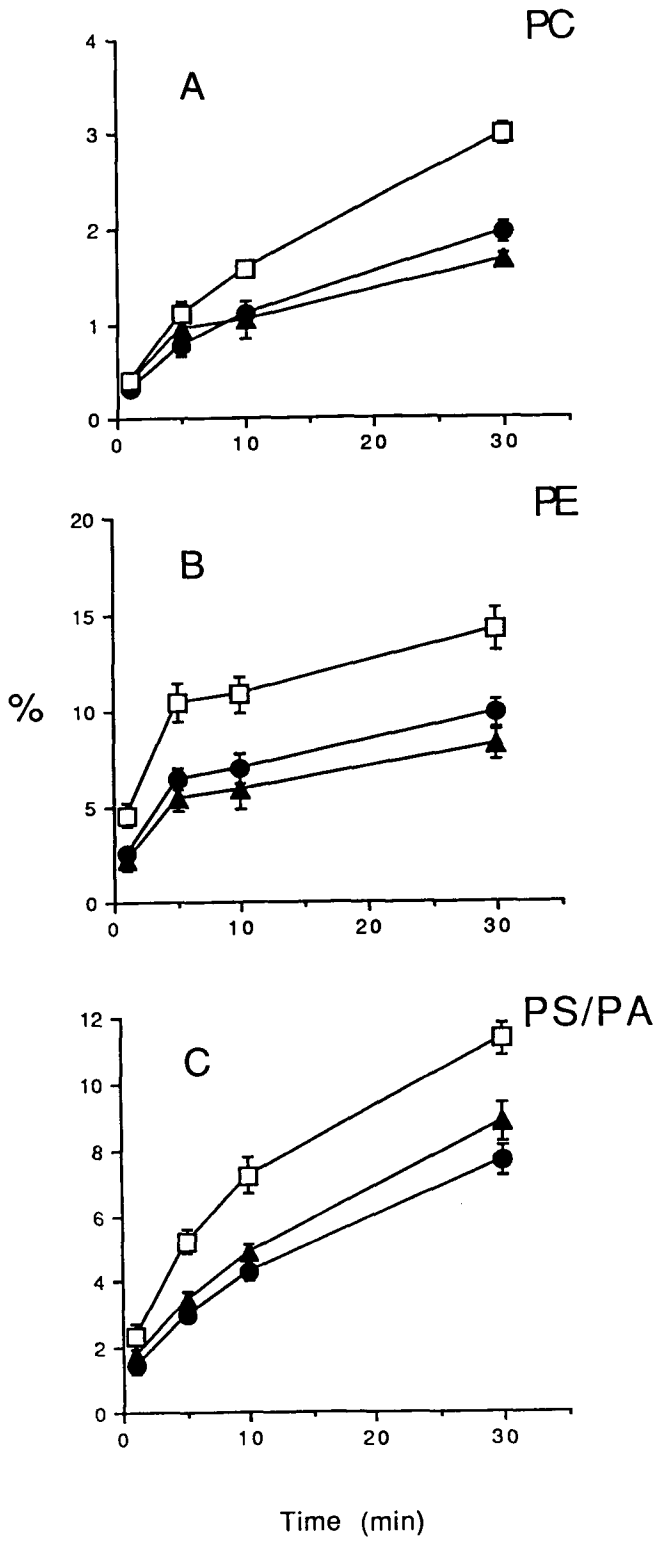


FIG. 1. Hydrolysis of PtdCho (A), PtdEtn (B), and PtdSer/PtdOH (C) containing 18:0 18:1, 18:0 20:4, and 18:0 22:6 species by cobra venom PLA_2. The average of three determinations is presented with standard error. Differences in hydrolysis of 22:6n-3 (○) and 20:4n-6 (●) at all data points were statistically significant at the $P < 0.01$ level; 18:1n-9 (▲).

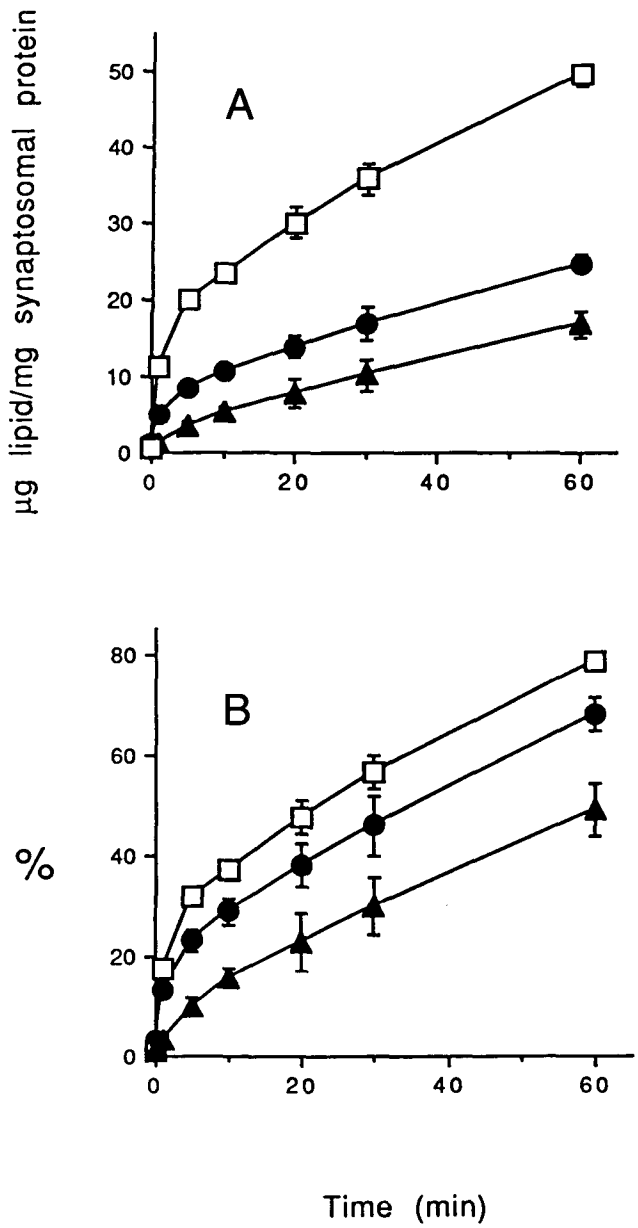


FIG. 2. Release of free fatty acids from rat brain synaptosomes by cobra venom PLA_2. Data points are presented as the absolute μg of released free fatty acid per mg synaptosomal protein (A) as well as the percentage against the total content of the corresponding fatty acid present in the incubated synaptosomes (B). The average of three determinations is presented with standard error. Differences in hydrolysis of 22:6n-3 and 20:4n-6 at all data points were statistically significant at the levels of $P < 0.001$ (all points in A), $P < 0.02$ (1, 5, 10, and 60 min points in B) and $P < 0.05$ (20 and 30 min points in B); ▲, 18:1n-9; ●, 20:4n-6, □, 22:6n-3.

in rat brain synaptosomes. Release of fatty acids by endogenous lipase activity from synaptosomes containing 0.125 mg protein was less than 10% of the hydrolysis observed with 0.1 unit cobra venom PLA_2 at any incubation time-point examined (Fig. 4). In contrast to the latter case, 20:4n-6 was found consistently at a higher level in comparison to 22:6n-3, despite the higher abundance of 22:6n-3 in synaptosomes. This hydrolysis pattern showing preferential release of 20:4n-6 was maintained in a wide range of pH values (5–9.5) and calcium concentrations (0.1 μM–100 mM), although maximum hydrolysis was achieved with 10 mM $CaCl_2$ at pH 9.

Cytosolic phospholipase inhibitors (18) partially inhibited the production of both 20:4n-6 and 22:6n-3. After 30 min of incubation, production of 20:4n-6 and 22:6n-3 fatty acids was slightly but significantly inhibited by 100 μM N-acetylneuraminic acid (NANA), 150 μM GM_3 ganglioside, and 100 μM N-glycolylneuraminic acid (NGN). Incubation with an extracellular PLA_2 inhibitor 4-bromophenacylbromide (0.5 μM) or a diacylglycerol (DG) lipase inhibitor protamin sulfate (0.5 μM) did not alter the extent of hydrolysis significantly, suggesting the possibility that a cytosolic PLA_2 may be involved in the hydrolysis process.

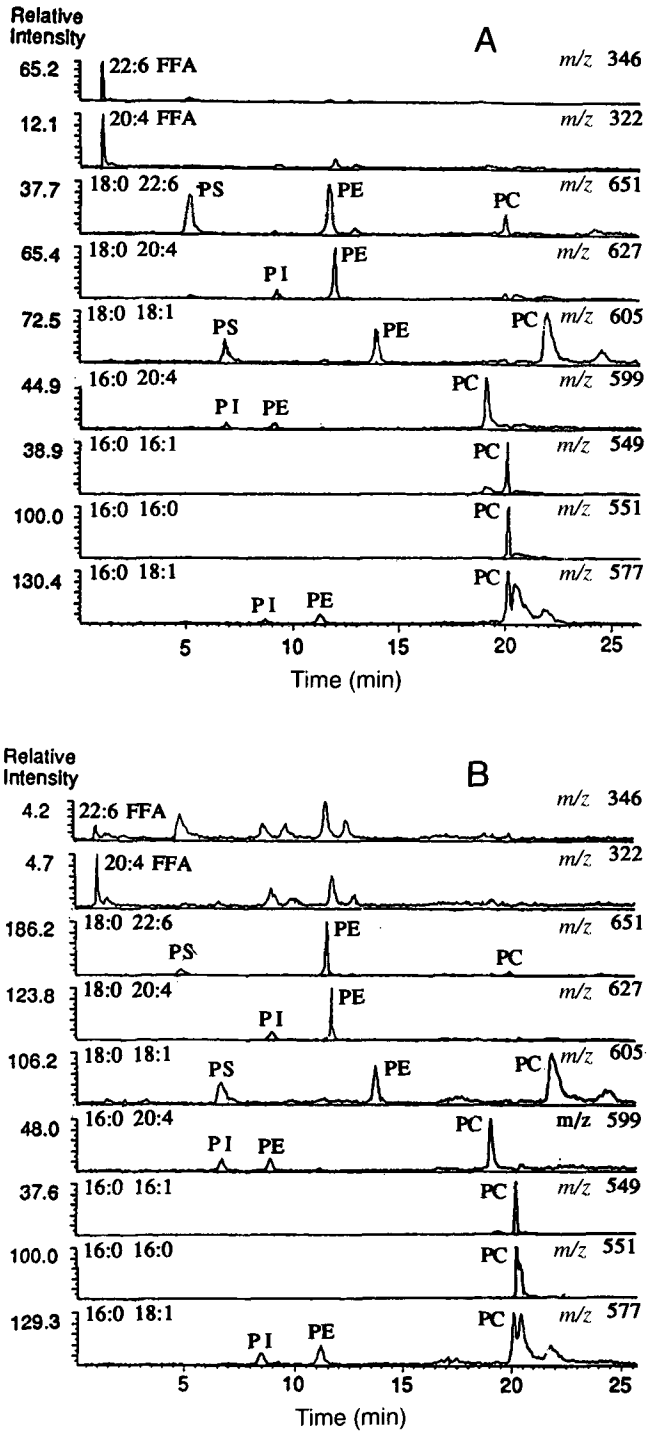


FIG. 3. Phospholipid molecular species analysis of rat brain synaptosomes before and after incubation with cobra venom PLA_2 for 1 min. Each molecular species was monitored by its diglyceride ion fragments, and relative intensity was calculated based on the area count of di-16:0-Gro*P*Cho species present in the synaptosomes. For free fatty acids, $(M + NH_4)^+$ ions were selectively monitored. Incubation time: A, 0 min; B, 1 min.

DISCUSSION

In this study, the substrate specificity of the cobra venom PLA_2 reaction was examined using lipid mixtures and rat brain synaptosomes. Results were compared with specificity observed for the synaptosomal fatty acid release by endogenous lipase activity. As indicated in data obtained with mixed lipids, cobra venom PLA_2 clearly showed substrate selectivity favoring 22:6n-3 for all PtdCho, PtdEtn, and PtdSer/PtdOH classes. Only 15 mol% of the mixed phospholipids (5 mol% each of 18:1n-9, 20:4n-6, and 22:6n-3 species) was introduced into the background lipids containing 40 mol% cholesterol, 25 mol% 16:0 16:1-GroPEtn, and 20 mol% di-palmitoyl-(di-16:0-) GroPCho. Therefore, the selectivity shown in this study did not appear to result from the different organiza-

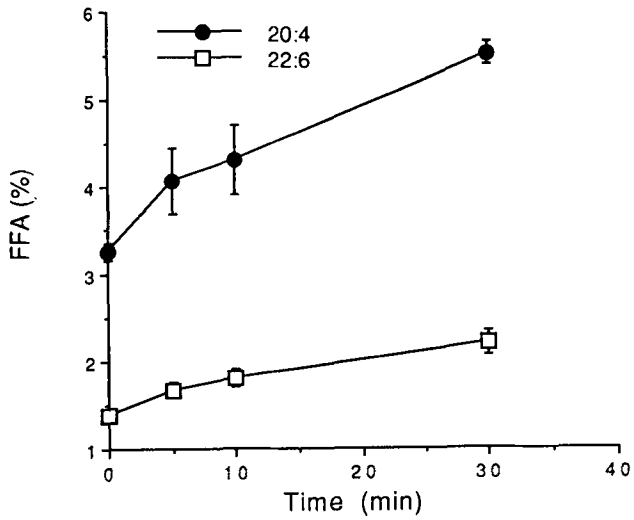


FIG. 4. Fatty acids released by endogenous lipase activity from rat brain synaptosomes. Data points are presented as the percentage against the total content of the corresponding fatty acid present in the incubated synaptosomes. The average of three determinations is presented with standard error; ●, 20:4; ■, 22:6.

tion of substrates at the lipid/water interface when substrates were presented to the enzyme (19). For lipid mixture and synaptosomes, hydrolysis rates were not linear, but gradually decreased after initial hydrolysis. This phenomenon may be explained by inactivation of PLA_2 during hydrolysis (20). Alternatively, product inhibition described for 85 kDa PLA_2 enzymes (21) may be considered. Due to the better affinity of the enzyme for lipid mixtures containing reaction products, free fatty acids, and lysophospholipids, the enzyme may become trapped and unavailable to other substrate vesicles. Our data obtained using mixed lipids or synaptosomes agreed that 22:6n-3 containing PtdEtn species were the best substrates, suggesting that the preferential release of 22:6n-3 is not just due to either the abundance of this species in synaptosomal PtdEtn or different efficiency in product inhibition or enzyme inactivation.

Preferential hydrolysis of 22:6n-3 by Naja naja naja PLA_2 observed in our work is similar to the findings from rat serum PLA_2 using PtdEtn liposomes obtained from rat brain synaptosomes (22). Selective hydrolysis of 22:6n-3 from rat brain synaptic membrane and myelin has been also observed from a group-II snake venom PLA_2 *Vipera russelli* (23). Since this selectivity disappeared when liposomes prepared with the lipid extract of either synaptic membranes or myelin were used as substrate (23), the authors attributed the observed selectivity to nonlipid components in membranes. Under our experimental conditions, however, the hydrolysis profile by Naja naja naja PLA_2 was similar for both lipid mixtures and synaptosomes in that 22:6n-3 containing PtdEtn species was the preferred substrate.

From our study it is clear that 22:6n-3 is selectively hydrolyzed from membranes of neuronal cells by Naja naja naja PLA_2. The selective loss of this species may cause impaired neuronal functions, as was suggested in the situation of n-3 deficiency (4,5). On the contrary, 22:6n-3 species are relatively resistant to endogenous lipase activity. While it is still not clear what the exact role of 22:6n-3 in neuronal membranes is, it is possible that the physiological importance may lie in its role as a membrane component rather than as a released free fatty acid.

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Natural and Accelerated Docosahexaenoic Acid Accumulation in the Prenatal Rat Brain

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ABSTRACT: The quantity and distribution of docosahexaenoic acid (DHA) in major brain phospholipids (PL) was examined in the fetal rat brain before birth, using thin-layer and capillary column gas chromatography. A rapid increment of DHA content of about 187 μg/g brain/day was observed between 17 to 20 days gestation, as opposed to 39.3 ± 2.9 μg/g brain/day prior to that. Single intraamniotic injections of 5 μL ethyl-docosahexaenoate (Et-DHA) (12 μM, 4.25 mg) administered to 17-day-old fetuses were used to examine the uptake of DHA into brain PL. Three days following injection, the amount of n-3 polyunsaturated fatty acids increased by 28% compared to ethyl-oleate (Et-Ole) injected fetuses. Compared to the n-6 fatty acid family, the relative amount of DHA increased in the phosphatidylserine (PS), phosphatidylethanolamine and phosphatidylcholine (PC) lipids by 15 ($P = 0.02$), 13 and 14%, respectively. A major increase in the pool size of phosphatidylinositol and PS (110 and 50.3%, respectively), and a decrease in PC (8.2%) were observed 3 d after Et-DHA as compared to Et-Ole administration. The data suggest that a single intraamniotic administration of Et-DHA can modulate membrane PL content and alter PUFA composition.

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Intrauterine life constitutes a vulnerable period in brain development, since the fetus is totally dependent upon the maternal supply of nutrients for its growth (1). The developing brain utilizes large amounts of long chain polyunsaturated fatty acids (PUFA), docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6) for the biosynthesis of rapidly expanding neuronal plasma membranes. Adequate dietary supply of PUFA on their precursors, linolenic and linoleic acids, is needed since these fatty acids cannot be synthesized *de novo* by mammals. Even though the rat brain growth spurt is considered a postnatal event (2,3), essential fatty acid (FA) deprivation during gestation leads to marked developmental deficits later in life (4) suggesting that significant PUFA changes occur also during pregnancy. However, data regarding these intrauterine changes in rat brain lipid FA are almost nonexistent.

DHA is found highly concentrated in brain tissue (5), but its specific role is still being investigated. To elucidate some of its functions, manipulation of brain DHA content, particularly DHA deprivation, mainly in postnatal animals, has been attempted (6). In some instances in which the fetal brain was examined, DHA content modification was done through the maternal feeding (7,8). These observations prompted us to examine alternative routes for direct fetal brain DHA modification, specifically enrichment, without the participation of the maternal metabolism. This work establishes the feasibility of the intraamniotic administration of ethyl-docosahexaenoate (Et-DHA) to accelerate the incorporation of DHA into the acyl chain groups of fetal brain phospholipids (PL). In addition, marked changes in the polar head-group composition of PL are documented.

MATERIALS AND METHODS

Pregnant Wistar (250–300 g body weight) rats were fed *ad libitum* chow containing 5 g lipid/200 g with the following fatty acid composition: 13% 16:0, 4% 18:0, 24% 18:1, 52% 18:2n-6 and 5% 18:3n-3. At days 17 and 20 of gestation (denoted E17 and E20), dams were anesthetized by intramuscular injection of 50 mg ketamine (Parke Davis, MI) and 7 mg xylizine HCl (Bayer, Leverkusen, Germany) per kg body weight (0.2 mL/300 g body weight of a 1:1 mixture of 2% xylizine HCl/Rompun and 0.1 g/mL Ketamine). After a midline abdominal incision, the uterine horns were exposed and kept moist throughout surgery with phosphate buffered saline (PBS, pH 7.4). For base line determination of brain lipids FA composition at either fetal age, the fetal brains were immediately removed upon midline incision of the abdomen and the uteri, and placed in liquid nitrogen. In the enrichment studies, E17 fetuses were injected intraamniotically through the uterine wall and amniotic sac with either 5 μL (12 μmols) Et-DHA (from Fish Oil Test Materials, NIAAA, NIH, Bethesda, MD) or an identical amount of ethyl-oleate (Et-Ole, Sigma, St. Louis, MO), both without any carrier. The uteri were returned to the abdomen, and 72 h later the fetuses were delivered by caesarean section. The fetal brains were immediately placed in liquid nitrogen upon removal.

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; Et-DHA, ethyl-docosahexaenoate; Et-Ole, ethyl-oleate; FA, fatty acid; GC, gas chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Pi, lipid phosphorus; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; TLC, thin-layer chromatography;.

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After weighing, the brains were homogenized in 2 mL hexane/isopropanol (3:2, vol/vol) containing 5 mg% butylated hydroxytoluene, using a Branson tip sonifier, according to Hajra and Radin (9). The organic layer containing the lipid extract was separated from the residual tissue by low speed centrifugation.

The major PL, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) were separated on thin-layer chromatography (TLC) by a mixture of chloroform/methanol/methylamine (40%) (130:70:30, by vol) (10). All the solvents were of HPLC grade.

Lipid spots resolved on TLC were visualized under ultraviolet light after spraying with 0.2% dichlorofluorescein in ethanol. Individual spots were identified and scraped into glass tubes and 1 mL of 14% BF_3 in methanol and 0.5 mL benzene were added. The tubes were capped under N_2 and heated at 100°C for 1 h. The methyl esters (FAME) were extracted into hexane after acidification of the mixture with 3 drops of 3N HCl and kept under N_2 at −20°C until analyzed. FAME separation was performed on a 3400 Varian GC using fused silica capillary columns [either SP-2330, 30 m × 0.25 mm inner diameter, 0.20 m film thickness (Supelco, Inc., Bellefonte, PA), or BPX70, 50 m × 0.22 mm inner diameter, 0.25 m film thickness (SGE, Melbourne, Australia)]. Column temperature was kept initially at 70°C for 1 min, then raised to 150°C at a rate of 20°C/min, further raised to 230°C at a rate of 3°C/min and after 14 min at this temperature, raised finally to 260°C at a rate of 20°C/min. Peak areas were integrated and plotted with the aid of the Varian Star Integrator computer package. FAME were identified by comparison of retention times with those of authentic standards (Nu-Chek-Prep, Elysian, MN; and Sigma Chemical, MO). Heneicosanoic acid (21:0, vol/vol) was used as internal standard for quantitation.

Lipid phosphorus was determined according to Bartlett (11).

Data were analyzed using the general linear model provided by the Statistical Analysis System (SAS) for ANOVA, nested design (12).

RESULTS

Maternal-dependent DHA accretion in the fetal brain. Total brain FA concentration increased 1.7-fold between E17 to E20 gestational age. The changes in the two essential FA family members, n-3 and n-6, however, did not proceed in parallel (Table 1). Whereas the relative concentration of the n-6 PUFA slightly decreased, that of the n-3 PUFA, consisting almost exclusively of DHA, increased substantially. Thus, the daily increment of DHA between E17 and E20 was on the average 187 μg/g brain/day. In contrast, the daily increment of DHA accretion up to E17 was on the average 39.3 ± 2.9 μg/g brain/day. This selective accumulation of DHA between E17 and E20 is reflected in the 1.5-fold increase in the DHA/total n-6 PUFA ratio (total n-6 PUFA is the sum of 20:4, 22:4 and 22:5) (Table 1).

TABLE 1
Fatty Acids of the Developing Fetal Rat Brain[a]

Parameters	E17	E20	*P*
Total FA (μg/mg wet wt)	8.80 ± 0.56	14.90 ± 0.54	0.0001
Total n-6 PUFA (wt%)	22.36 ± 0.40	19.78 ± 0.32	0.02
Total n-3 PUFA (wt%)	7.08 ± 0.36	9.86 ± 0.35	0.0001
DHA (wt%)	6.43 ± 0.36	8.55 ± 0.39	0.0001
DHA/total n-6 LC-PUFA	0.31 ± 0.02	0.46 ± 0.01	0.03

[a]Mean ± SE, n = 6 for E17, each sample of three brains, n = 8 for E20, each sample of two brains. total n-6 polyunsaturated fatty acids (PUFA) include 18:2, 20:3, 20:4, 22:4, and 22:5; total n-3 PUFA include 18:3, 22:5 and 22:6; total n-6 long-chain (LC)-PUFA include 20:4, 22:4, and 22:5. DHA, docosahexaenoic acid.

The increase in DHA, relative to total n-6 PUFA, was noted in all major brain PL studied. As illustrated in Figure 1, there was a 1.4–1.7-fold increase in the DHA/total n-6 PUFA ratio between E17 and E20, highly significant in the PS and PE lipid fractions.

Fetal brain DHA accretion in the Et-DHA supplemented fetuses. To investigate the effect of Et-DHA supplements on the normal acquisition of DHA in the fetal brain, selected fetuses were injected on day 17 of pregnancy as detailed under methods. This mode of injection did not cause any premature deliveries. After three days following injection into the amniotic sac, all fetuses survived, suggesting that the route and amounts of Et-DHA or Et-Ole injected were not toxic.

Analysis of several FA parameters in the fetal brain at E20 is summarized in Table 2. There were no significant differences in the total brain FA concentration between the Et-DHA and Et-Ole injected fetuses. The total brain n-3 PUFA and DHA proportions increased by 28.3 and 20.7%, respectively, after Et-DHA as compared to Et-Ole administration (P = 0.009 and 0.024, respectively). There was a 47% increase of the DHA/total n-6 PUFA ratio following Et-DHA as compared to Et-Ole administration (P = 0.01). The changes of this ratio in the individual PL are depicted in Figure 2. Moderate increases of 14, 15 (P = 0.02), and 13% were observed in PC,

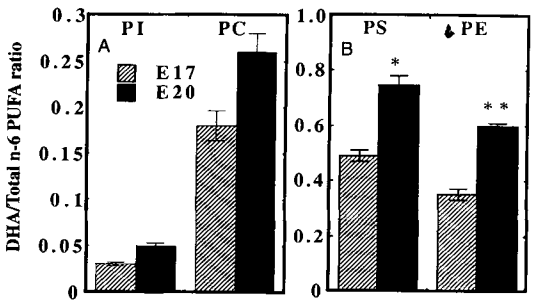


FIG. 1. Developmental changes in fetal rat brain phospholipid (PL) docosahexaenoic acid (DHA) and n-6 polyunsaturated fatty acid (PUFA). The ratios of brain PL DHA to the sum of the long-chain n-6 PUFA (20:4, 22:4, and 22:5) in the 17-d (E17, hatched bars) and 20-d (E20, dark bars) gestational age fetuses are shown. Values are mean ± SE of 4–8 samples, each E17 sample consisting of three brains and each E20 sample of two brains. *P = 0.005; **P = 0.0001.

TABLE 2
Effects of Intraamniotic Ethyl-Docosahexaenoate (Et-DHA) Administration[a]

Parameters	Et-Ole	Et-DHA	*P*
Total FA (μg/mg wet wt)	13.84 ± 0.63	13.40 ± 0.41	NS
Total n-6 PUFA (wt%)	21.85 ± 0.56	20.57 ± 0.22	NS
Total n-3 PUFA (wt%)	8.61 ± 0.22	11.05 ± 0.34	0.009
DHA (wt%)	8.55 ± 0.30	10.32 ± 0.30	0.02
DHA/total n-6 LC-PUFA	0.41 ± 0.003	0.53 ± 0.02	0.01

[a]Mean ± SE, n = 6 for ethyl-oleate (Et-Ole), n = 14 for Et-DHA, each sample of two brains. See footnotes to Table 1 for abbreviations and other information.

PS, and PE, respectively, following Et-DHA administration. A slight reduction of 7% was noticed in PI.

Since the FA changes observed in the total brain extracts were not matched by the changes in the individual PL fractions, an alternative explanation related to the pool size of the brain PL was considered. As illustrated in Figure 3, three days after Et-DHA administration, a significant increase of more than twofold in PI content ($P = 0.0009$) was observed. Similarly, PS increased by more than 50% ($P = 0.03$) and PC decreased by 8.2% ($P = 0.03$). The change in PE was not significant. Under these conditions, total brain lipid phosphorus (Pi) of Et-DHA and Et-Ole injected fetuses was similar (0.88 ± 0.03 μg/mg, and 0.83 ± 0.03 μg/mg, respectively).

DISCUSSION

After cessation of neuronal cell proliferation, the last several days before birth in the rat are characterized by an active growth and acquisition of new plasma membranes as well as the formation of stable connections between neuronal cells. The strikingly accelerated rate of DHA accretion during the last three days of pregnancy documented in this study is presumably maintained by the maternal diet. We suggest that a "growth spurt" for DHA accumulation exists, which is analogous to the traditional concept of the "brain growth spurt" coined by Dobbing (2), but significantly antedates it. This prenatal period, during which the fetus is entirely dependent on the maternal diet, is very vulnerable to nutritional insults

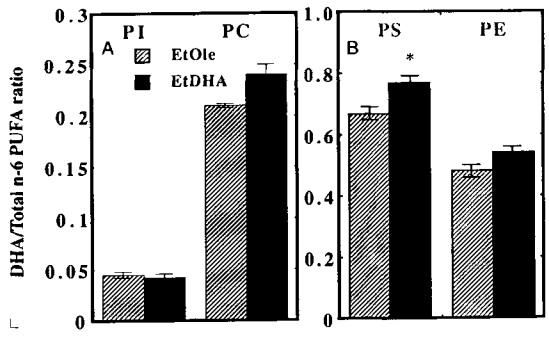


FIG. 2. Effects of ethyl-docosahexaenoate (Et-DHA) administration on the fetal rat brain PL DHA and n-6 PUFA. Symbols and abbreviations as in Figure 1, except that each sample consisted of two fetal brains. *P = 0.02. Et-Ole, ethyl-oleate.

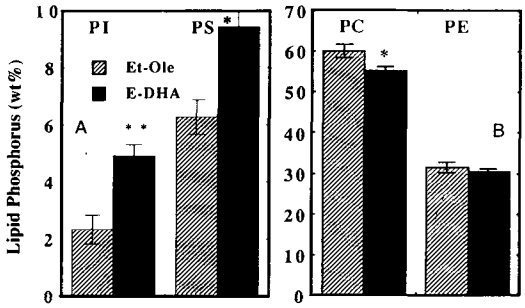


FIG. 3. Effects of Et-DHA administration on the fetal rat brain PL polar head group composition. Symbols and abbreviations as in Figures 1 and 2. *$P < 0.05$; **$P < 0.02$.

in general and to deficiencies in the DHA supply in particular, leading to possible pathological consequences in the neonate (2,4).

The major aim of this work has been to assess the effectiveness of the intraamniotic route to accelerate the incorporation of DHA into fetal brain PL. Although the intraamniotic injection is an invasive approach, the ability to rapidly enhance DHA esterification during intrauterine life may hold potential clinical value whenever an indication for DHA deficiency exists, such as possible prematurity (13).

Our approach of direct administration of pure DHA (as its ethyl-ester) turned out to be advantageous over previous dietary approaches using oil mixture supplements (8,14–18). Most of these diets contain various combinations of DHA and eicosapentaenoic acid (EPA, 20:5n-3), which cause a decrease in n-6 PUFA after prolonged administration. This undesirable consequence of DHA enrichment (19,20) has been attributed mainly to the presence of EPA in the fish-oil supplement (21). Pure Et-DHA administration by the intraamniotic route, on the other hand, did not impair the levels of AA and was associated with only a slight, nonsignificant decrease in all the n-6 PUFA, in spite of a 21% increase in the brain DHA concentration. The ethyl ester formulation has repeatedly been used for DHA and other FA administration and is effectively absorbed and incorporated into tissues (22–25). Delivery of PUFA by the intraamniotic route has been done, to our knowledge, for the first time in the present study.

Another point of interest is the elevation of PI and PS amounts as a result of Et-DHA administration. While information pertaining to the intrauterine brain DHA accretion in the rat is scant (26), data on the FA composition in individual PL and the changes during this period are not available. Under natural, maternal feeding conditions, we show most pronounced changes in DHA composition in the PS and PE lipids. Since PE is quantitatively more abundant than PS, more of the brain DHA is esterified in the former. However, even though PS is a relatively minor PL quantitatively, it may be functionally very important (27). It is conceivable that failure to attain the proper FA composition, especially DHA in PS, may be the crucial factor in the functional deficits resulting from dietary alterations (27).

Concomitant with the accelerated DHA accumulation, we clearly demonstrate an increase in the PS content in brain tissue, most likely as a result of base exchange activity. Since serine base exchange is the only accepted pathway for PS synthesis in mammals (28,29), it would appear that Et-DHA administration enhanced the activity of this enzyme. The increase in fetal brain PI content following Et-DHA administration is presumably accomplished by enhanced synthesis via phosphatidic acid precursor, a hypothesis which needs to be examined.

In summary, we have demonstrated that a single intraamniotic administration of ethyl-docosahexaenoate to 17-day fetal rats caused both DHA-enrichment of fetal brain lipids and substantial changes in their polar-head composition 72 h later. These data suggest that at least part of the actions of this essential FA is through modulation of membrane PL content. This method of rapid modulation of DHA in fetal tissues should be instrumental in furthering our understanding of the role(s) of DHA in cell function.

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n-3 Polyunsaturated Fatty Acids and Human Cytokine Synthesis

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ABSTRACT: The cytokines interleukin-1 and tumor necrosis factor-α are potent peptide mediators of the immune response. They exert potent cellular effects at nanomolar concentrations with target tissues ranging from the vascular endothelium, to the central nervous system and immunocompetent cells. The synthesis of these two cytokines can be suppressed pharmacologically by steroids and other immunosuppressive agents such as cyclosporin A. In previous studies we have found that such an inhibition of inflammatory cytokine synthesis can also be achieved by increasing the content of n-3 polyunsaturated fatty acids in leukocyte membranes.
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The cytokines interleukin-1 and tumor necrosis factor (TNF-α) contribute to the pathogenesis of inflammatory diseases and, potentially, atherogenesis. This lends importance to our original observation that oral supplementation of omega-3 or n-3 fatty acids to diets of healthy volunteers suppresses the capacity of mononuclear cells (MNC) to synthesize interleukin-1 and TNF-α. In recent years, this finding has been extended in several directions: (i) the suppression of cytokine synthesis by n-3 fatty acids can also be observed in patients with an inflammatory disease, i.e., rheumatoid arthritis, (ii) the suppression of cytokine synthesis can also be achieved by an alteration of the diet without using lipid supplements, and (iii) suppression of cytokine synthesis is probably achieved on the level of transcription, as evidenced by decreased levels of mRNA. The mechanism by which n-3 fatty acids exert suppression of cytokine synthesis remains unknown. The effect may, however, contribute to the beneficial effects of n-3 fatty acids observed in models of chronic inflammatory disease.

The cytokines interleukin-1 and tumor necrosis factor. Cytokines are secreted proteins with central paracrine functions in inflammation and proliferation. They comprise the families of interleukins, TNF-α, interferons, and the hematopoietic growth factors. Among this large group of proteins, a subgroup initially identified as products of monocytes have a similar profile of biological activities. This group comprises: the two forms of interleukin-1, interleukin-1α, and interleukin-1β, TNF-α and, to a certain extent, interleukin-6. Among the many biological activities shared by these cytokines, their actions on endothelial cells appear to be of particular importance in the pathogenesis of atherosclerosis. Of these, interleukin-1 potentiates procoagulant acitivity, and increases production of plasminogen activator inhibitor and endothelin, as well as the formation of eicosanoids. Furthermore, it increases leukocyte adhesion by inducing the expression of adhesion molecules, and it promotes endothelial protein permeability.

Aside from their pyrogenic effects, interleukin-1 and TNF-α influence a wide array of biological functions (Fig. 1) (1). Many of the biological functions of interleukin-1 are shared by TNF-α (2).

The study of the interleukin-1 system has gained additional momentum by the discovery of the interleukin-1-receptor antagonist. This protein was identified as an endogenous inhibitor of interleukin-1 action. It shares sequence homologies with interleukin-1α and interleukin-1β and binds to the same receptor as these two proteins, but has no intrinsic activity. Aside from its enormous biological significance, the interleukin-1-receptor antagonist has proved an excellent experimental tool in demonstrating the necessary role of interleukin-1 in certain disease processes (3). Mitigating disease in animal models by adminis-

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Abbreviations: TNF-α, tumor necrosis factor-α; MNC, mononuclear cells.

FIG. 1. Biological Activities of Interleukin-1. Figure courtesy of Dr. Jos W. M. van der Meer, Nijmegen, The Netherlands.

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FIG. 2. Influence of n-3 fatty acids on production of interleukin-1β stimulated with endotoxin (10 ng/mL). Nine healthy volunteers added 18 g of fish oil concentrate (MaxEPA®) per day to their normal diet. *In vitro* production of interleukin-1β was determined by incubating peripheral blood mononuclear cells for 24 h with different stimuli. At the end of the incubation, the cells were lysed by freeze-thawing to obtain the total, that is, cell-associated plus secreted cytokine. Interleukin-1β was measured by specific radioimmunoassay. The columns represent the mean values for nine volunteers. Bars indicate standard error of the mean. *Indicates significant difference from the baseline (before n-3 diet) at $P < 0.05$.

tration of exogenous interleukin-1-receptor antagonist proves participation of interleukin-1 as a necessary intermediate. This approach has been used in several disease models.

The potent and diverse biological functions of cytokines such as interleukin-1 and TNF-α place particular importance on the mechanisms of their regulation and the question of targeted modulation. The synthesis of cytokines is tightly controlled by a host of extracellular factors, plasma membrane constituents and cytosolic and nuclear factors. An example of differential regulation is the pharmacological elevation of intracellular cAMP. This intervention markedly suppresses synthesis of TNF-α without affecting the synthesis of interleukin-1 (4).

Modulating the synthesis of interleukin-1 and tumor necrosis factor by n-3 polyunsaturated fatty acids. Pharmacologic agents known to reduce the synthesis of interleukin-1 and TNF-α synthesis are corticosteroids and cyclosporine A. Since interleukin-1 and TNF-α are principal mediators of inflammation, reduced production of these cytokines may contribute to the amelioration of inflammatory symptoms in patients taking n-3 fatty acid supplementation. Therefore, we have investigated the effects of n-3 fatty acids on the synthesis of the cytokines interleukin-1 and TNF-α (5).

Figure 2 illustrates the production of interleukin-1β during the course of the study. Our findings demonstrate that n-3 fatty acid supplementation reduced the ability of MNC to produce interleukin-1β upon stimulation with endotoxin. The effect was most pronounced 10 wk after stopping the supplementation and suggests prolonged incorporation of n-3 fatty acids into a pool of circulating MNC. The capacity of the MNC from these donors to synthesize interleukin-1β returned to the presupplement level 20 wk after ending supplementation. Similar results were observed for interleukin-1α and TNF-α.

The findings form a pathophysiologic rationale for therapeutic trials with n-3 fatty acids in certain diseases, e.g. rheumatoid arthritism, with documented involvement of inflammatory cytokines, such as interleukin-1 and TNF-α. Furthermore, a suppression of the magnitude we observed can only be achieved pharmacologically by administration of glucocorticoids or cyclosporine A, which have well-known adverse side effects, particularly during long-term administration.

In an animal transplant model, n-3 fatty acids even enhanced cyclosporine A-induced immunosuppression (6). In 1993, the intriguing results of a one year intervention trial with dietary fish oil in 66 patients after renal transplantation were reported (7). In this randomized, double-blind study, the daily administration of 6 g of fish oil (containing 3 g of n-3 fatty acids) had a beneficial effect on renal hemodynamics and on blood pressure in renal transplant recipients treated with cyclosporine. Furthermore, the fish-oil group had significantly fewer rejection episodes than the control group and there was a trend to increased graft survival. If these positive results are confirmed in a larger patient group, they may alter the treatment of choice for primary prevention of transplant rejection.

Other studies on the modulation of cytokine synthesis by n-3 fatty acids. Suppression of interleukin-1 and TNF-α synthesis by n-3 fatty acids has been found in other studies *in vivo* in humans (8–12). The study design and results are compared in Table 1. Of particular interest is the most recent study by the group of Meydani who, for the first time, studied the effect of n-3 fatty acids in dietary intervention (rather than as encapsulated lipid supplement) on cytokine synthesis. After a 24-wk diet that provided 1.2 g n-3 fatty acids per day, lipopolysaccharide-induced synthesis of interleukin-1β was decreased by 40%.

Recently, studies in animal models have been reported that dietary n-3 fatty acids did not inhibit, but markedly enhanced lipopolysaccharide-stimulated production of either TNF-α (13), or both interleukin-1α and TNF-α (14). In Table 2, the results of animal studies *in vivo* on the influence of n-3 fatty acids on cytokine synthesis are summarized. Interestingly, the three studies in which enhancement of cytokine synthesis was found were all performed using peritoneal macrophages from mice. In contrast, in the study by Billiar *et al.* (15) the interleukin-1 and TNF-α synthesis of Kupffer cells from rats was suppressed to an extent similar to that observed in humans. Whether this discrepancy within animal models represents a difference in the cell population studied or a difference in the species is not clear at present (16).

The mechanisms by which n-3 fatty acids affect cytokine synthesis remain unclear. However, studies demonstrating reduced levels of mRNA for platelet-derived growth factor (17) and for interleukin-1β (18) point to regulation at a pretranslational level. The decrease of interleukin-1β synthesis together

TABLE 1
Effect of n-3 Fatty Acids on Cytokine Production in Humans[a]

Source by	Model	Cells	n-3 Fatty acid dose duration	Suppression	
Endres *et al.* (5)	Healthy volunteer	MNC	4.6 g/d × 6 wk	IL-1 61% ↓	TNF 40% ↓
Kremer *et al.* (8)	Patients with rheumatoid arthritis	MNC	6.7 g/d × 24 wk	IL-1 41% ↓	
Meydani *et al.* (9)	Healthy volunteer	MNC	2.4 g/d × 12 wk	IL-1β 50% ↓	TNF 52% ↓
Mølvig *et al.* (10)	Healthy volunteer, diabetics	MNC	3.2 g/d × 7 wk	IL-1β 40% ↓	TNF noΔ
Meydani *et al.* (11)	Healthy volunteer	MNC	1.2 g/d × 24 wk	IL-1β 40% ↓	TNF noΔ
Cooper *et al.* (12)	Healthy volunteer	Whole blood	1.2 g/d × 6–8 wk	IL-1[b] 85% ↓	TNF[b] no Δ

[a]MPh, macrophages; MNC, mononuclear cells; TNF, tumor necrosis factor; only cell-associated.
[b]Only secreted.

with decreased formation of interleukin-2 also translates into reduction of a functional parameter of lymphocyte activation, i.e., phytohemagglutinin-induced proliferation (19).

Conclusions. The results of this and other studies demonstrate that oral supplementation with n-3 fatty acids can markedly suppress the synthesis of interleukin-1 and, to a smaller extent, TNF-α by human MNC. Given the potent effects of these cytokines in the pathogenesis of atherogenesis and inflammation, these results argue for cytokine suppression as a mechanism contributing to the protective role of n-3 fatty acids in atherosclerosis and certain inflammatory diseases. Given the potentiating effects of both cytokines on target tissues, this finding may be even more important for effects *in vivo*. Future research in this field will focus on three questions: (i) What is the intracellular mechanism for the inhibition of cytokine synthesis? As mentioned above, reduction of cytokine mRNA points to an effect of n-3 fatty acids at the level of transcription or increased degradation; (ii) What is the precise constituent of n-3 fatty acid containing fish oil concentrates that mediates the effect? All studies on n-3 fatty acids and cytokine synthesis have used concentrated fish oil preparations. It will be of particular interest to find out whether a particular fatty acid, such as eicosapentaenoic or docosahexaenoic acid, is responsible for the effect; (iii) What is the reason for the *increase* in cytokine synthesis after feeding n-3 fatty acids that has been observed in some animal models? Some evidence points to a species or cell population difference because increase in cytokine synthesis was always observed in mouse peritoneal macrophages. One may speculate that either the preparation of the peritoneal macrophages or the immunological compartment renders the cells activated to a certain extent.

As to therapeutic recommendations it is important to stress that studying the effect of n-3 fatty acids on cytokine synthesis does not allow us to draw conclusions on clinical end points. These conclusions remain to be drawn from controlled intervention studies in patient groups that are complete or are currently in progress. However, when searching for the mechanism of clinically demonstrated beneficial effects, the cytokine system is a highly attractive area of mediating mechanisms.

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TABLE 2
Animal Studies *in vivo* Examining the Effect of n-3 Fatty Acids on Cytokine Production[a]

Source of TNF	Model	Cells	n-3 Fatty acid duration	Change IL-1
Billiar *et al.* (15)	Rats	Kupffer cells	6 wk	IL-1 ↓ TNF ↓
Lokesh *et al.* (16)	Mouse	Peritoneal macrophages	4 wk	IL-1 ↑ TNF ↑
Hardardottir *et al.* (13)	Mouse	Peritoneal macrophages	5 wk	IL-1 ↑
Blok *et al.* (14)	Mouse	Peritoneal macrophages	6 wk	IL-1 ↑ TNF ↑

[a]Abbreviation as in Table 1. IL, interleukin.

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Effects of Modulation of Inflammatory and Immune Parameters in Patients with Rheumatic and Inflammatory Disease Receiving Dietary Supplementation of n-3 and n-6 Fatty Acids

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ABSTRACT: To describe the rationale and status of n-3 and n-6 fatty acid dietary supplementation in patients with inflammatory disease. The most recent literature is reviewed with a focus on rheumatoid arthritis (RA) as most investigations have described the use of n-3 supplements in this disease entity. Investigations from Europe, the United States, and Australia have described consistent improvement in tender joint scores with many investigators also observing improvements in morning stiffness. A meta analysis has confirmed the predictable improvement in tender joints. Recent studies also suggest that some patients with RA are able to discontinue nonsteroidal antiinflammatory drugs (NSAIDs) while receiving n-3 fatty acids. A large number of peer reviewed publications from around the world have established the utility of dietary supplementation with n-3 fatty acids in reducing tender joint counts and morning stiffness in patients with RA. Some patients are also able to discontinue NSAIDs while on these supplements.
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Due to the mammalian inability to interconvert n-3 and n-6 fatty acids, the composition of phospholipids in cellular membranes is determined by nutritional intake (1). The fatty acids found in the membrane bilayer are substrates for the production of prostaglandin and leukotriene species essential for many biological activities including modulation of the inflammatory and immune response. It is becoming widely recognized that reproducible metabolic alterations in these pathways can be engineered by consistent modifications in dietary fatty acid content. These fatty acid induced changes have been documented in animal (2), and normal humans trials (3) and large epidemiological studies of populations consuming relatively homogeneous diets (4).

Investigations have examined the effects of dietary fatty acid supplementation in a variety of autoimmune diseases. As most of these studies have occurred in patients with rheumatoid arthritis, we will focus primarily on this disease, while briefly reviewing the results in other inflammatory disease processes. We will review the reported effects of both n-3 and n-6 fatty acids.

n-6 Fatty acids. γ-Linolenic acid (GLA) (18:3, n-6) is found in seeds from the evening primrose and borage plants. It may be converted by an elongase enzyme to dihomogammalinolenic acid (DGLA) (20:3, n-6). DGLA is oxidized by cyclooxygenase to prostaglandin E_1 (PGE_1), a monoenoic prostaglandin which has altered biologic activities from the dienoic prostaglandin PGE_2. For example, PGE_1 can inhibit platelet aggregation in humans and rats *in vivo* and *in vitro*, whereas PGE_2 cannot (5). PGE_1 dietary supplementation can suppress inflammation and joint tissue injury in several animal models of inflammation (6) and can inhibit neutrophil function. GLA dietary supplementation can suppress acute and chronic inflammation in several experimental animal models.

The subcutaneous air pouch model of inflammation was used to assess response to monosodium urate crystals or Freund's adjuvant in Sprague-Dawley rats consuming diets enriched with either safflower oil as a source of linoleic acid or borage seed oil as a source of GLA (7). Animals consuming GLA had a marked reduction of neutrophil exudate and lysosomal enzyme activity compared with those fed sunflower oil. PGE_2 and leukotreine B_4 (LTB_4) concentrations were significantly diminished in pouch exudates in the GLA fed rats. The fatty acid profiles in the serum and inflammatory cells form animals consuming primrose oil exhibited significant increases in GLA and DGLA.

When DGLA is added to human synovial cells grown in tissue culture interleukin-1B (IL-1B) stimulated growth is suppressed fivefold compared with cells grown in medium supplemented with arachidonic acid (8). Cells incubated with DGLA exhibited a 14-fold increase in PGE_1, and a 70% decrease in PGE_2 compared with cells in control medium. The increase in PGE_1 concentrations was associated with significantly enhanced levels of cAMP which the authors suggested was responsible for the antiproliferative effects. The inhibiting effect could be blocked by indomethacin, lending further

Abbreviations: DGLA, dihomogammalinolenic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; IL-1B, interleukin-1B; LTB_4, B_5, leukotreine B_4, B_5; NSAID, nonsteroidal antiinflammatory drug; PAF, platelet activating factor; PGE_1, E_2, E_3, prostaglandin E_1, E_2, E_3; RA, rheumatoid arthritis; TNF, tumor necrosis factor.

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support to PGE_1 mediation of growth suppression.

Human studies. An open study of only 20 patients with rheumatoid arthritis treated with GLA in combination with various vitamins showed no significant changes of any clinical or laboratory parameters over a period of 12 wk (9). An investigation comparing evening primrose oil, as a source of GLA, to olive oil in 20 patients with rheumatoid arthritis over a period of 13 weeks failed to show any significant difference between groups (10).

In a well designed study, investigators treated patients with rheumatoid arthritis with 1.4 g of GLA daily over period of 24 wk (11). They observed a clinically important reduction in both the tender and swollen joint counts of 36 and 28%, respectively, while the placebo group showed no improvement in these parameters. It had previously been demonstrated that GLA can inhibit IL-2 production by peripheral blood mononuclear cells *in vitro* (12) and may reduce the expansion of activation markers on T lymphocytes (13). The mechanism of the improvements observed was not studied (11).

n-3 Fatty acids. Eicosapentaenoic acid (EPA) can be metabolized through both the cyclooxygenase and 5-lipoxygenase metabolic pathways to form end-products with altered biological activity. The beneficial effects of these alterations on the cardiovascular system will be discussed elsewhere. EPA is metabolized to PGE_3, a compound with less inflammatory activity than PGE_2 (14). PGE_2 stimulates osteoclast activity resulting in bone resorption, a process of significance in rheumatoid arthritis. The dienoic prostaglandins also increase vascular permeability in a synergistic effect with serotonin and bradykinin (14). The E-series prostaglandins are not localized to specific tissues as is the case with thromboxane and prostacyclin which are limited to platelets and vascular endothelium respectively. Therefore, the effect of altering PGE content in any specific site will vary depending upon the tissue where the synthesis takes place (15).

The effects of n-3 fatty acids in altering eicosanoid metabolism are of great importance in immune and inflammatory disease. They can inhibit the synthesis of arachidonic acid from its linoleic acid substrate (16) possibly through competition for the 2-position occupied by arachidonate in membrane phospholipids. EPA will also directly compete with arachidonic acid as the substrate for cyclooxygenase and inhibit its metabolism into eicosanoids (17). n-3 Fatty acid ingestion thus results in the production of compounds with altered biological activity as well as a decrease in the usual amounts of biologically active arachidonate derivatives.

Ingestion of EPA is associated with the production of leukotrienes with 5 double bonds such as LTB_5 which has greatly attenuated proinflammatory activities (18). LTB_5 is usually undetectable in humans consuming a Western diet. Simultaneous with the production of small amounts of LTB_5 from stimulated neutrophils of normal subjects consuming fish oil, there are large decreases in the production of neutrophil LTB_4 (19). Neutrophil chemotaxis was significantly suppressed in these subjects after six weeks of fish oil ingestion and returned to normal after the supplements were discontinued.

Other investigators have found evidence of a selective inhibition of the epoxide hydrolase enzyme in neutrophils from normals (18) and rheumatoid arthritis patients (20) consuming fish oil because of unaltered quantities of 5-hydroxyeicosatetraenoic acid and 5-hydroxyeicosapentaenoic relative to the corresponding arachidonic acid product generated before dietary supplementation.

Chemotactic activity of neutrophils from rheumatoid arthritis patients who had ingested fish oil was found to be enhanced compared to the pretreatment state (20). This is seemingly paradoxical in view of the suppression of neutrophil chemotaxis after fish oil ingestion in normals (19). The improved chemotaxis in rheumatoid arthritis patients represents a partial correction of a reduced chemotactic activity of peripheral blood neutrophils of subjects with this disease. Platelet activating factor-acether (PAF-acether) generation of stimulated monocytes is significantly diminished (19) after fish oil ingestion, suggesting a n-3 fatty acid induced inhibitory effect of phospholipase A2 activity on substrate alkyl phospholipids which would affect PAF generation. This is of considerable interest in the context of inflammation in that PAF can stimulate tumor necrosis factor (TNF) as well as other cytokines in endothelial tissue (21) and is 1,000 times more potent than histamine in inducing changes in vascular permeability (22). Evidence suggests that PAF can also modulate T cell and monocyte function (23).

n-3 Fatty acid ingestion is associated with significant changes in the fatty acid profiles of cellular lipids. Neutrophil arachidonic acid content is reduced by 33% and EPA content is enhanced twenty-fold compared to presupplement values obtained in rheumatoid arthritis patients ingesting fish oil (20). n-3 Fatty acid ingestion is thus associated with reproducible changes in the biochemical composition of cell membranes of neutrophils and monocytes and striking alterations in the production of the metabolic end products of a myriad of inflammatory and immunologically active compounds which may influence the course of rheumatic disease.

Animal studies. Dietary modifications containing n-3 fatty acids reduce the severity of diffuse proliferative glomerulonephritis in several autoimmune strains of mice including the NZBxNZWF1, BxSB/Mpj and MRL/lpr strains (24,25). Dietary n-3 fatty acids reduce the severity of glomerulonephritis even when they are withheld until after the renal disease has begun to evolve (24). Not all observed effects of fish oil have been beneficial in the laboratory: an increased incidence of arthritis was observed in rats immunized with type II collagen who consumed a fish oil-enriched diet compared with animals ingesting beef tallow although the severity of the arthritis was the same in each group (25). An increased incidence of necrotizing vasculitis was noted in renal arteries of MRL/lpr mice with systemic lupus erythematosus-like disease, even while their glomerulonephritis was alleviated on a fish oil diet (24). Studies indicate that a mixture of n-3 fatty acids containing both EPA and docosahexaenoic acid may be more effective than either of these n-3 fatty acids by themselves (26).

Human studies. The effects of dietary fish oil supplements in patients with rheumatoid arthritis have been studied in several investigations (27–39). Studies on mostly Caucasians of Northern European background have been reported from the United States (27–31), Australia (32) and Europe (33–39). It is unlikely that background dietary habits were uniform in these populations with a highly varied geographic distribution. Some populations were known to consume a higher content of fish in their diet (36) than others (40). An improvement in the number of tender joints on physical examination is most often observed (28,30–35) with some authors reporting improvement in Ritchie Articular Index (35,38), and morning stiffness (30,32,35). Omega-3 fatty acid supplements have been studied in comparison with a variety of dietary interventions including inert paraffin wax (27), corn oil (31,35), olive oil (28–30), and a specially prepared mixture of fatty acids designed to reproduce the local dietary intake (36,38). The benefits in the number of tender joints and morning stiffness have been confirmed in a recent meta-analysis (41).

It is accurate to portray the overall clinical response to fish oil supplements reported in these investigations as modest (42,43). It is clear, however, that dietary fatty acids produce reproducible alterations in eicosanoid metabolism which should aid in the amelioration of inflammation (28,29,32,44, 45,46). In addition, fish oil supplements have been associated with a decreased production of interleukin-1B in normals (47) and patients with rheumatoid arthritis (28,30). It is the unique ability of dietary fatty acids to alter cell membrane fatty acid constituents (42) which has given rise to interest in the potential of these dietary substances to influence an array of immune parameters.

In a recently completed investigation (31), we were interested in expanding our observations of the effects of fish oil in patients with rheumatoid arthritis to determine if patients consuming high dose supplements would be better able to discontinue their non-steroidal anti-inflammatory drug compared with a control population given corn oil. Sixty-six patients entered a prospective double-blind investigation. Patients were randomized to receive fish oil ingested 130 mg/kg/d of n-3 fatty acid (ethyl ester concentrate prepared by the National Marine Fisheries Association for the National Institute of Health), while control patients consumed corn oil capsules. All patients were given the nonsteroidal anti-inflammatory drug diclofenac throughout the study and this was replaced with a placebo diclofenac, while patients continued their fatty acid supplements. Clinical evaluations were performed for several months after discontinuing the diclofenac and also after stopping the fish oil and corn oil supplements. A variety of cytokines were measured throughout the study including interleukin-1, 2, 6 and 8 and TNF-α.

Our results again showed that fish oil dietary supplementation results in significant improvement in tender joints and other clinical parameters of disease from baseline (31). However, none of the improvements in the patients receiving fish oil achieved significance at the time of the maximum duration of diclofenac therapy at 18 or 22 wk when compared with patients on corn oil. Patients receiving corn oil also exhibited many improvements during this time interval which did not achieve statistical significance. In addition, the magnitude of the improvement from baseline that we observed in patients on high dose fish oil was indistinguishable from that previously reported when patients consumed total doses of n-3 fatty acids of from 3–6 g/d (28,30). We cannot therefore recommend further investigations with the doses we employed which resulted in the ingestion of 9 g of daily n-3 supplements in a 70 kg individual.

Improvements from baseline in patients with rheumatoid arthritis consuming fish oil often do not achieve statistical significance vs. other dietary fatty acid interventions. This may be because the biological effects are not powerful enough or alternatively due to either a placebo effect or real biological effects induced by the so-called placebo fatty acids. We have previously wrestled with the issue of an ideal control fatty acid to compare with fish oil (30) and chose corn oil in our most recent investigation (31) after using olive oil (28,30). It is possible that any mono or polyunsaturated fatty acid has significant potential immune effects. We believe that the issue of the ideal placebo dietary intervention to compare with fish oil in the study of autoimmune inflammatory disease has not yet been settled.

We examined the clinical status of patients after discontinuing diclofenac while receiving fish oil and corn oil in several ways (31). We examined patient clinical status of diclofenac after the maximal duration of fish oil exposure (weeks 26 or 30) and compared this with their baseline status while receiving diclofenac. The improvement in tender joints was significant in the patients remaining on fish oil at this time when compared with both baseline status and with patients on corn oil during the same period. We again observed a significant decrease in IL-1, confirming our previous observations (28,30), but did not find significant decreases in IL-2, 6, 8 or TNF-α.

Others have reported upon whether fish oil dietary supplements can affect nonsteroidal antiinflammatory drug (NSAID) requirements in patients with rheumatoid arthritis (35,37,48). Skoldskam *et al.* used an open patient directed NSAID reduction and found that patients consuming fish oil were able to significantly reduce their NSAID dose compared with a control group. Belch *et al.* used an open, physician-directed NSAID reduction while subjects consumed only 240 mg EPA along with 450 mg of GLA and found that after one year subjects were able to significantly reduce these medications (37). Kjeldson-Kragh *et al.* (35) substituted placebo Naproxen for real drug in a stepwise manner. Because of the study design, evaluators in this investigation knew that there was a 67% likelihood that patients had discontinued Naproxen. Nevertheless, they reported that patients on fish oil tolerated stopping NSAID better than controls. Finally, Lau *et al.* (48) recently reported that patients on fish oil were able to significantly reduce their NSAID requirements compared with a control group. We believe that our recent data (31) support the previous observations that selected individu-

als with rheumatoid arthritis may discontinue NSAID therapy while consuming n-3 supplements.

Other inflammatory conditions. Subsequent to the reports of benefits of fish oil supplementation in patients with rheumatoid arthritis, n-3 supplements have been employed with some reported benefit in patients with inflammatory bowel disease (49) and most recently in IgA nephropathy (50). It is apparent that many other inflammatory conditions could hypothetically benefit from the n-3 induced changes in eicosanoid metabolism and decreased production of IL-1 previously described. It is likely that we will see many more studies examining the effects of n-3 supplements in inflammatory disease in future years.

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The Significance of Polyunsaturated Fatty Acids in Cutaneous Biology

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ABSTRACT: The skin epidermis displays a highly active metabolism of polyunsaturated fatty acids (PUFA). Dietary deficiency of linoleic acid (LA) and 18-carbon (n-6) PUFA results in characteristic scaly skin disorder and excessive epidermal water loss. Arachidonic acid, a 20-carbon (n-6) PUFA is metabolized via the cyclooxygenase pathway into predominantly prostaglandin E_2 (PGE_2) $PGF_{2\alpha}$, and PGD_2 and via the lipoxygenase pathway into predominantly 15-hydroxyeicosatetraenoic acid (15-HETE). The prostaglandins modulate normal skin physiological processes at low concentrations and inflammatory reactions at high concentrations. Similarly, the very active epidermal 15-lipoxygenase transforms dihomogammalinolenic acid (DGLA) into 15-hydroxy eicosatrienoic acid (15-HETrE), eicosapentaenoic acid (EPA) into 15-hydroxyeicosapentaenoic acid (15-HEPE) and docosahexaenoic acid (DHA) into 17-hydroxydocosahexaenoic acid (17-HDoHE), respectively. These monohydroxy acids exhibit anti-inflammatory properties. In contrast, the 18-carbon (n-6) PUFA is transformed into 13-hydroxy-9,11-octadecadienoic acid (13-HODE), which exerts antiproliferative properties in the tissue. Thus the supplementation of diets with appropriate purified vegetable oils and/or fish oil may generate local cutaneous anti-inflammatory metabolites which could serve as a less toxic *in vivo* monotherapy or as adjuncts to standard therapeutic regimens for the management of skin inflammaory disorders.
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The nutritional significance of certain polyunsaturated fatty acids (PUFAs) was heightened when Burr and Burr (1,2) reported that rats maintained on a fat-free diet over a long period developed abnormalities characterized by growth retardation, severe scaly dermatosis of the dorsal and pedal skin, caudal necrosis and extensive water loss through the skin. Prominent in this deficiency syndrome is a defective barrier to water loss (3). Because these deficiency symptoms, notably the cutaneous symptoms, could be reversed by certain dietary PUFAs, they were designated *essential fatty acids* (EFAs).

Dietary sources. The 18-carbon n-6 and n-3 PUFAs are synthesized on land by many plants and therefore are obtained from vegetable oils. However, the longer chain members of each family are either biosynthesized *in vivo* after dietary ingestion of the shorter 18-carbon precursors or they are obtained directly from animal or marine sources. For example, the longer chain n-3 PUFAs, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are found in fish and marine oils and therefore can be ingested directly from these sources. The important longer chain n-6 PUFA, arachidonic acid (AA, 20:4n-6), is found in liver, brain, and muscle, which are rich dietary sources of arachidonic acid.

Significance of 18-carbon polyunsaturated acids in the epidermis: (i) *The role of linoleic acid (18:2n-6) in epidermal water barrier systems.* A specific and essential function of the 18-carbon linoleic acid (18:2n-6) is its involvement in the maintenance of the epidermal water barrier (4) which is one of the epidermal abnormalities of cutaneous EFA deficiency. This significant effect of linoleic acid (LA) was reported early in a series of studies by Prottey and co-workers who demonstrated that increased transepidermal water loss occurred in EFA-deficient animals (5). Information derived from these findings supported the special role of LA in restoring the defect of epidermal water barrier which has been induced by EFA-deficiency. In later reports, the physical structure of the epidermal water barrier was described to be sheets of stacked lipid bilayers or lamellae which fill the intercellular spaces of the uppermost layer of the epidermis (stratum corneum). These lipid bilayers contain large amounts of sphingolipids (6) of which the linoleate-rich species have been characterized as acylglucosylceramide, acylceramide and a unique acylacid (7,8).

(ii) The role of linoleic acid in hyperproliferative processes. The suggestion that the linoleyl moiety of the barrier acylsphingolipids is further metabolized by a lipoxygenase-like reaction to form first a hydroxyacylceramide and then a polyoxyacylceramide prior to modulating the barrier systems (9) stimulated great interest in developing a mechanism for

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Abbreviations: AA, arachidonic acid; DGLA, dihomogammalinolenic acid; DHA, docosahexaenoic acid; EFA, essential fatty acid; EFAD, essential fatty acid deficient; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid; 13-HODE, 13-hydroxy-9, 11-octadecadienoic; 13HODE-DAG, 13-HODE–containing diacylglycerol; LA, γ-linoleic acid; LTB_4, leukotriene B_4; PGE_2, prostaglandin E_2; PKC, protein kinase C; PMN, polymorphonuclear cell; PUFA, polyunsaturated fatty acid.

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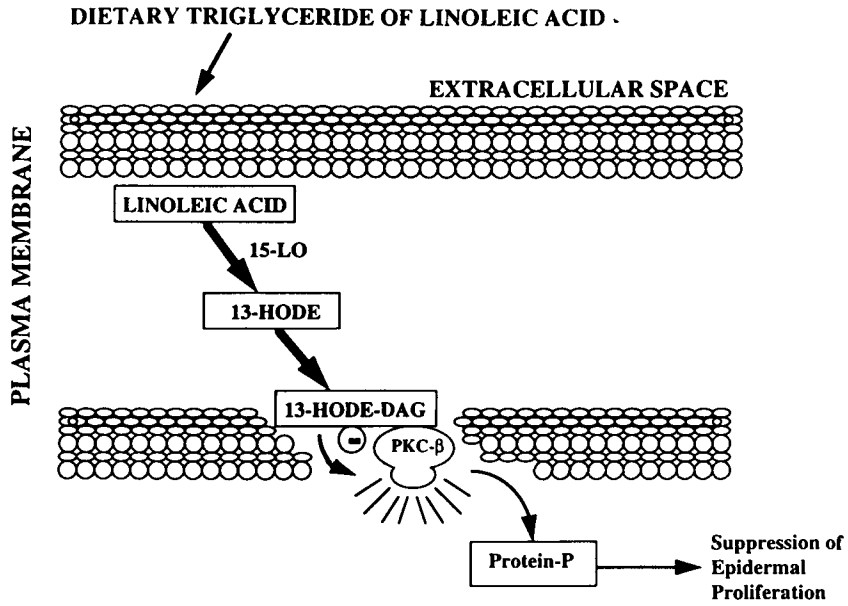


FIG. 1. Speculative scenario of dietary linolenic acid modulation of epidermal hyperproliferation via down-regulation of protein kinase C (PKC)-β. 13-HODE, 13-hydroxy-9, 11-octadecadienoic acid; 13-HODE–DAG, 13-HODE containing diacylglycerol.

linoleic acid action. The above finding prompted these investigators to propose that restoration of the defective epidermal water barrier system depended upon the formation of these oxygenated species. The view is consistent with the presence of a very active 15-lipoxygenase activity in the epidermis which converts LA (18:2n-6) into predominantly a 13-hydroxy-9, 11-octadecadienoic acid (13-HODE) (10). It seems very likely that this oxygenated metabolite is the suggested active molecular form of LA in the epidermal water barrier study.

(iii) Functional role of 13-hydroxyoctadecadienoic in the epidermis. The incubation of LA with 15-lipoxygenase prepared from human epidermis yields a major metabolite, 13-hydroxy-9, 11-octadecadienoic acid (13-HODE) (10). Interestingly, our first speculation of a possible functional role of 13-HODE arose from our observation that epidermal levels of 13-HODE were suppressed after the induction of epidermal hyperproliferation. The topical application of 13-HODE to a guinea pig model of epidermal hyperproliferation resulted in the reversal of the hyperproliferation to a normal state (11). To delineate a possible mechanism for the effect of topical 13-HODE, we demonstrated that topical 13-HODE was incorporated first into the inositol phospholipids followed by a phospholipase C-catalyzed release into 13-HODE-containing diacylglycerol (13HODE-DAG) (12). This novel 13-HODE-substituted-DAG was markedly depleted in the hyperproliferative skin in EFA deficiency, paralleling the increased activities of epidermal protein kinase C-α (PKC-α) and PKC-β. Replenishment of the hyperproliferative epidermis with topical 13-HODE resulted in the accumulation of tissue 13HODE-DAG, the selective suppression of PKC-β activity (13) and the reversal of the epidermal hyperproliferation to normal.

To establish the *in vivo* relevance of these observations, guinea pigs were made essential fatty acid deficient (EFAD). Tissue levels of putative 13HODE-DAG, PKC-isozymes and tissue hyperproliferation were determined during the development and reversal of EFAD. Findings from these experiments revealed that cutaneous 13-HODE and novel 13HODE-DAG were markedly lower in EFAD animals than in LA-enriched safflower oil-fed control animals. These reductions paralleled both the elevated EFAD-induced epidermal hyperproliferation, and the elevated expressions and activities of PKC-α and β-isozymes as determined by Western Blot. Refeeding the animals with LA/γ-linolenic (GLA) enriched safflower oil for two weeks replenished the *in vivo* tissue level of 13HODE-DAG that paralleled both the selective down regulation of PKC-β expression and activity and the reversal of hyperproliferation. These results suggest that an elevated level of epidermal 13-HODE plays a role in modulating cutaneous hyperproliferation *in vivo* via the generation of 13HODE-DAG and the selective suppression of PKC-β isozyme. A speculative scenario of the possible modulating effects of dietary LA/13-HODE on epidermal inflammatory/proliferative processes is illustrated in Figure 1.

The role of 20-carbon polyunsaturated fatty acids in the epidermis—Metabolism and functional roles: Cyclooxygenase pathway. The 20-carbon AA is the second prominent PUFA in the skin. Its functional role depends largely on the generation of biologically potent oxidative metabolites. Arachidonic accid represents approximately 6–10% of the total fatty acids in the epidermal phospholipids of guinea pigs (14). Similarly, human epidermal phospholipids contain approximately 9% AA (15). Specific epidermal enzymes metabolize AA into a variety of oxygenated metabolites, which in turn exert a variety of functions on the skin. One

major function is that AA metabolites regulate proliferative and/or differentiating processes in the epidermis. The epidermal AA when released undergoes oxidative transformations by either the cyclooxgenase to generate mainly prostaglandins E_2 (PGE_2), $PGF_{2\alpha}$, and PGD_2, or lipoxygenases which in this tissue generate mainly hydroxy fatty acids.

Functionally, the prostaglandins play a central and complex role in inflammation and therefore are pertinent to the pathogenesis and treatment of cutaneous inflammatory diseases. For example, in early reports, repeated injections of PGE_2 were reported to cause a persistent swelling of the rodent paw which interfered with ambulation. Similarly, repeated injections of PGE_1/PGE_2 into human skin resulted in cutaneous inflammatory reactions characterized by erythema, edema, and tenderness. The beneficial effects derived from administration of nonsteroidal anti-inflammatory drugs under these conditions did implicate some of these prostanoids in the cutaneous inflammatory processes.

(ii) Lipoxygenase pathway. The lipoxygenase pathway is very active in the epidermis. While the epidermal tissue or the epidermal cells (keratinocytes) lack the 5-lipoxygenase and thus cannot transform precursor arachidonic acid directly into leukotriene B_4 (LTB_4), it does, however, possess an active LTA_4 hydrolase and can transform polymorphonuclear cell (PMN)-derived leukotriene A_4 (LTA_4) into LTB_4. Thus, conversion of LTA_4 into LTB_4, can be catalyzed by epidermal LTA_4-hydrolase (16,17). The above possibility may explain the elevated LTB_4 in PMN-infiltrated cutaneous psoriatic plaque, thus, contributing to the immense skin proliferation in this desease.

The epidermal lipoxygenase enzymes also catalyze the generation of hydroxy fatty acids from a variety of polyunsaturated fatty acids. Oxygenation of arachidonic acid by epidermal 12-lipoxygenase yields 12-hydroxyeicosatetraenoic acid (12-HETE). Advances in chiral phase chromatography now reveal that 12-HETE which originally was reported to accumulate in the lesion of psoriatic patients is primarily the 12(R) epimer and not the 12(S) epimer that is the typical 12-lipoxygenase product formed by platelets.

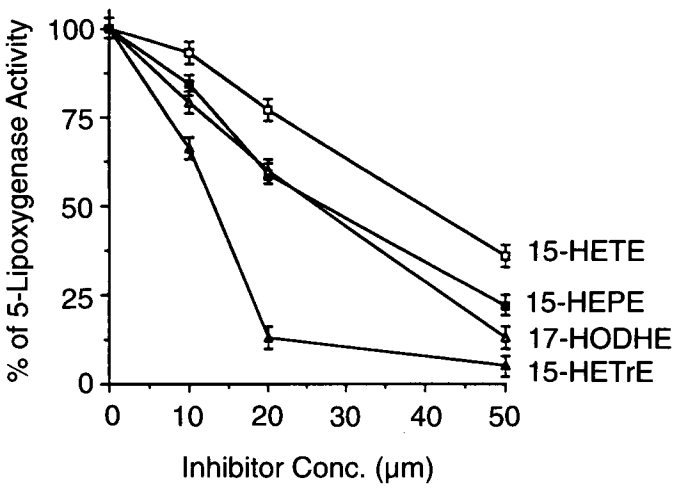


FIG. 2. Comparative effects of monohydroxy fatty acids derived from arachidonic acid, □; eicosapentaenoic acid, ■; docosahexaenoic acid, △; and dihomogammalinolenic acid, ▲.

The 15-lipoxygenase enzyme is very active in the epidermis. Metabolic products of a variety of PUFAs are generated via this pathway that exert potent biological effects in this tissue. For example, 15-HETE, a product derived from AA, has been demonstrated in human epidermis, in cultured murine and human epidermal cells (keratinocytes), in isolated human neonatal foreskin and in guinea pig epidermis. This monohydroxy fatty acid was detected in biologically active concentrations in human patients with psoriatic lesions (18). Functionally, 15-HETE was reported to inhibit 5-lipoxygenase activity and generation of LTB_4 in neutrophils and basophils *in vitro* (19). These *in vitro* effects suggest, at least in part, the anti-inflammatory potential of 15-HETE. Consistent with this anti-inflammatory potential, 15-HETE was reported to improve the symptoms of psoriasis vulgaris after intralesional injections (20).

Oxygenation of other polyunsaturated fatty acids via *15-lipoxygenase pathway*: *(i) The incubation of GLA with epidermal preparation results in its rapid elongation into dihomogammalinolenic acid (DGLA).* Similarly, dietary supplementation of guinea pig or human diet with GLA-enriched oils results in marked elevation of DGLA in the tissue. The guinea pig and human epidermis, contain a very active elongase that catalyzes the conversion of precursor GLA into DGLA (21). The DGLA, on the one hand, is metabolized by epidermal cyclooxygenase into prostaglandin of the 1-series (PGE_1), on the other hand, it is metabolized by epidermal 15-lipoxygenase into 15-hydroxyeicosatrienoic acid (15-HETrE) (22) which is a potent inhibitor of LTB_4 generation *in vitro*. When guinea pig diet was supplemented with GLA-enriched oil, 15-HETrE was generated *in vivo* in the epidermis (23). Similarly, when the human diet was supplemented with GLA-containing capsules, a significant amount of DGLA was found in the PMN phospholipids. The elevation of DGLA paralleled the decreased capacity of isolated PMNs to biosynthesize LTB_4 (24).

(ii) Metabolism of EPA (20:5n-3) and DHA (22:6n-3). The *in vitro* incubations of EPA and DHA with epidermis results in the formation of two 15-lipoxygenase products: 15-hydroxyeicosapentaenoic acid (15-HEPE) and 17-hydroxydocosahexaenoic acid (17-HODHE) respectively (25). When guinea pig diet was supplemented with EPA/DHA-enriched fish oil, marked elevation of 15-HEPE and 17-HODHE were generated in the epidermis (26).

Taken together, the 15-lipoxygenase catalyzed monohydroxy fatty acids generated from the variety of 20-carbon PUFAs are potent inhibitors of LTB_4 generation from polymorphonuclear cells. Comparative inhibitory effects of these monohydroxy fatty acids are illustrated in Figure 2. The data reveal dose-dependent *in vitro* effects of the monohydroxy acids derived from AA, EPA, DHA, and DGLA on LTB_4 generation by rat basophilic leukemic-1 (RBL-1) cells. 15-HETrE notably derived from DGLA is the most potent at 20 μM.

Modulation of epidermal eicosanoid generation: (i) Pharmacological effects. Pharmacological inhibitors of

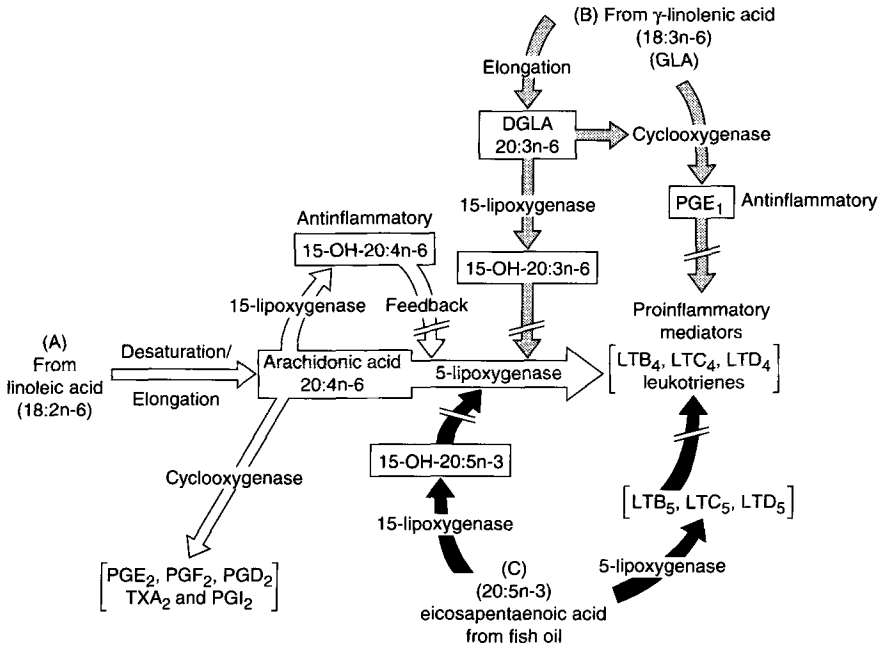


FIG. 3. A speculative scenario of the possible modulatory effects of the constituent fatty acids and metabolites from vegetable and fish oils on the generation of proinflammatory leukotrienes from arachidonic acid.

proinflammatory prostaglandins and leukotriene synthesis, as well as the antagonists of other endogenously biosynthesized inflammatory mediators, have become important front line agents or adjuncts in the treatment of a variety of inflammatory conditions. However, their uses have been limited in cutaneous inflammatory disorders. A number of 5-lipoxygenase inhibitors and antagonists selective for specific leukotrienes have been synthesized, but their efficacy for the management of inflammatory/proliferative skin disorders remains to be fully demonstrated.

(ii) Dietary effects of PUFA. Since the precursor of the variety of eicosanoids are derived from dietary PUFA, it is reasonable to expect that the *in vivo* synthesis of the mediators can be modulated by manipulation of the diets. Thus the supplementation of diets of psoriatic patients with EPA/DHA-enriched fish oil resulted in the alleviation of skin inflammatory/proliferative processes (27–29). The results of these dietary studies are compatible with the view that diets enriched with EPA and DHA exert profound effects on endogenous epidermal phospholipid fatty acid composition, on the endogenous release of AA and the biosynthesis of the inflammatory eicosanoids. For instance, the supplementation of guinea pig diet results in an epidermal increase of DGLA, which in turn parallels the increase in the levels of epidermal 15-HETrE (a 15-lipoxygenase metabolite of DGLA) (18).

A speculative scenario of the possible modulatory effects of the constituent fatty acids and metabolites from vegetable and fish oils on the generation of proinflammatory leukotrienes from AA is shown in Figure 3. The [A] pathway illustrates the metabolic transformations of dietary LA which results in the tissue generation of 15-HETE and proinflammatory leukotrienes by PMNs. The [B] pathway represents the epidermal transformation of dietary GLA (e.g. from primrose oil and/or borage oil) into DGLA and the tissue generation of 15-HETrE (via 15-lipoxygenase pathway) and PGE_1 (via the cyclooxygenase pathway). The [C] pathway represents the metabolic transformations of dietary EPA into 15-HEPE and LTB_5 from EPA. It is, therefore, reasonable to speculate that metabolites from both the [B] and [C] pathways could inhibit the *in vivo* generation of local tissue proinflammatory leukotrienes from AA. These possibilities suggest that the dietary intake of highly purified vegetable or fish oils, containing appropriate constituent PUFAs from these oils, may offer an alternative or adjunct therapeutic modality for alleviating cutaneous inflammatory disorders.

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Membrane Docosahexaenoic Acid vs. Eicosapentaenoic Acid and the Beating Function of the Cardiomyocyte and Its Regulation Through the Adrenergic Receptors

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ABSTRACT: The β-adrenergic system *in cardiac* muscle cells is influenced by the n-3 polyunsaturated fatty acid (PUFA) content in cell membranes. This study deals with the specific effect of docosahexaenoic acid (DHA) as compared to other n-3 PUFA, without modification of the arachidonic acid content. Increasing the DHA content in the phospholipids of isolated cardiomyocytes did not provoke electrical or contraction modifications, except for a slightly lower plateau phase (–2 mV). Conversely, the β-adrenergic function was affected at several levels: (i) the receptor affinity for dihydroalprenolol tended to decrease (Kd) without alteration of the number of β-binding sites (β_{max}); (ii) the isoproterenol-induced (10^{-7} M and 10^{-6} M) cAMP production was significantly decreased (–20%); and (iii) the positive chronotropic response to β-adrenergic stimulation (isoproterenol, 10^{-7} M) was significantly enhanced (+80%). In order to further investigate the relationship between the decreased cAMP and the increased chronotropic response, the cells were treated with dibutyryl-cAMP, a permeant analogue of cAMP, which elicited a significantly higher chronotropic response in DHA-enriched cells than in EPA-enriched cells (+50%). The increase in DHA content in cardiac cell membranes phospholipids may thus affect the β-adrenergic system through an increase of cAMP efficiency. Although the membrane phosphatidylinositols were largely involved in the PUFA alterations, none of the parameters related to α-*adrenergic* system (chronotropic response, receptor density, affinity for prasozin, and inositide phosphate production) were influenced by the membrane DHA content.
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The beneficial effects of fish oil consumption in reducing the incidence of heart disease have been attributed to their high content in long-chain n-3 polyunsaturated fatty acids (PUFA), mainly eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3; DHA) (1–3). Most of these beneficial effects were attributed to EPA, through its various vascular and serum consequences, including platelet aggregation, vasodilatation, and circulating lipids (4,5). Recently, the protective effect of fish oils was suggested to rely on mechanisms other than those related to their vascular influences (6). Recent studies have shown that a fish oil diet may increase the contractility of the left ventricle and improve myocardial relaxation (7). But little information is available on the direct effects of the long-chain n-3 PUFA on the cardiac function at the muscle cell level. The age-dependent changes in calcium channel density and affinity were reported to be altered by fish oil diets (8). Conversely, the influence of n-3 fatty acid supplementation of myocardial cells on the activity of Ca^{++} pump (9) and Na^{+} pump (10) is controversial. In addition, the incorporation of EPA into the membrane phospholipids (PL) of cultured cardiac myocytes appeared to protect the cells from the toxic effect of ouabain by lowering the Ca^{++} overload (11). In a previous paper, we reported that the modification of the phospholipid n-6/n-3 fatty acid ratio had no influence on the basal electrical and mechanical activities in isolated ventricular myocyte (12). Moreover, there is increasing evidence that the function of adrenergic receptors in the heart is influenced by changes in membrane PL composition (13). However, there are few studies on the specific effect of n-3 PUFA on reactivity to autonomic neurotransmitters. Some data were obtained in clinical studies, suggesting a relationship between dietary-induced lipid modifications and the cardiovascular response to noradrenaline (14). Similarly, the influence of fish oil diets on noradrenaline sensitivity, through alterations in the receptor affinity and density (15–18), adenylate cyclase (19), and/or cyclic nucleotide phosphodiesterase (20) have been reported. From the various studies published to date, it is difficult to conclude whether the effects of fish oil diets on the heart are due to a decrease in membranes n-6 PUFA or to a specific effect of the increase in DHA. The functional alterations specifically induced by the membrane enrichment in DHA remain largely unknown, although fish oil diets result in a large increase of DHA in heart membranes with little or no variation in EPA (21). Therefore, the present study deals with the consequences on ventricular myocytes of increasing membrane content in DHA without decreasing the arachidonic acid content.

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PL, phospholipids; PUFA, polyunsaturated fatty acids.

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MATERIALS AND METHODS

Cell culture. Rat ventricular myocyte cultures were prepared as previously described (22). The cells were grown in a standard culture medium [Ham's F10 (Seromed Biochrom, Berlin, Germany) medium supplemented with 10% fetal calf serum and 10% human serum] in a humidified atmosphere at 37°C containing 5% CO_2. After 24 h, the cells were incubated for four days in the same medium supplemented as serum albumin-bound fatty acids with either 20:5n-3 (EPA) or 22:6n-3 (DHA), which led to two populations (EPA and DHA cells) from the same cell pool (23).

Fatty acid analysis. The lipids were extracted according to Folch *et al.* (24). PL were separated from nonphosphorus lipids on silica gel cartridges (25). The lipids were transmethylated with using BF_3–methanol and the fatty acid methyl esters were analyzed by gas chromatography.

Physiological parameters. The experiments were carried out in custom made Puck's F salt solution under a paraffin oil layer (26). The intracellular voltage measurements were done using conventional microelectrodes. The cell contractions were monitored using a custom-made motion detector (27). The adrenergic stimulation was carried out as described elsewhere (27). The drugs used were: l-isoproterenol HCl (Iso; Sigma, St. Louis, MO), dibutyryl adenosine 3′,5′-cyclic monophosphate (dBcAMP), and phenylephrine (Phe; Sigma).

Messenger production. The β-adrenergic receptors were stimulated for 3 min by Iso in the presence of 3-isobutyl-1-methylxanthine (10^{-3} M). The reaction was stopped by heat shock and the intracellular cAMP content was determined by radio immunoassay (Amersham, Slough, United Kingdom). The α-adrenergic receptors were stimulated by addition of Phe, after radio labelling of phosphatidylinositol with [^{3}H]-inositol. The phosphoinositide breakdown was expressed by the accumulation of [^{3}H]-inositol-1-phosphate (Ins1P) in the presence of lithium (28,29). The method of Lowry *et al.* (30) was used for protein content measurements.

Receptor binding. The number of receptor binding sites and their affinity were measured on a whole cell homogenate obtained from a pool of 50 dishes per group. The β- and α-adrenergic receptor density was determined using [^{3}H]-dihydroalprenolol (0.15–3.0 nM) and [^{3}H]-prazosin (32–640 pM) (DuPont, NEN, Wilmington, DE), respectively. The β- and α-adrenergic receptor binding was assessed by measuring the amount of bound ligand displaced by alprenolol d-tartrate (1 μM) and phentolamine (10 μM), respectively.

Statistics. The results are presented as mean ± SEM. The data were submitted to analysis of variance (31). The differences were considered significant for $P < 0.05$.

RESULTS

Fatty acid composition. The fatty acid composition of the PL of EPA- and DHA-treated cells is shown in Figure 1. The total amounts of saturated fatty acids, monounsaturated fatty acids, and PUFA were similar in the two groups. The membrane PL of the DHA cell group were characterized by a large incorporation of DHA (approximately 15%) with little incorporation (less than 4%) of EPA and its elongation product, docosapentaenoic acid (22:5n-3). In contrast, the PL fraction of the EPA cell group contained large amounts of both EPA and docosapentaenoic acid (approximately 10% each) and only 1.5% of DHA. All these differences in n-3 PUFA contents were significant ($P < 0.001$). Conversely, the two cell groups displayed a similar content in arachidonic acid, as the n-6 PUFA composition was not significantly altered.

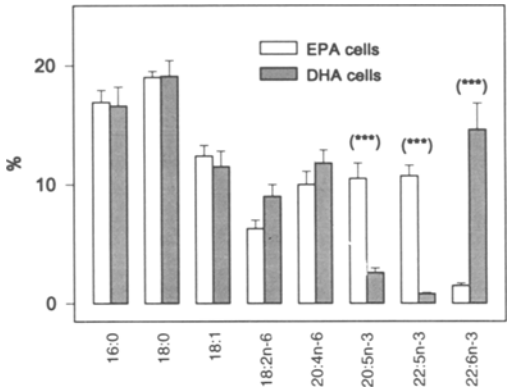


FIG. 1. Fatty acid composition of the phospholipid fraction of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) cells (as % of total phospholipid fatty acid). (***), $P < 0.001$.

Physiological properties. The amplitude of the action potential in the EPA-treated cells was slightly but significantly higher than in the DHA cells (Table 1). Conversely, no significant difference could be observed in the maximal diastolic potentials. This difference in action potential amplitude could be mainly attributed to a significantly less positive plateau potential level (–2.1 mV) in the DHA cells (see OS in Table 1). In contrast, all the time- and rate-parameters of the action potential (Table 1) were independent of the membrane DHA level. Similarly, the contraction parameters, such as duration at 80% relaxation (CD80), contraction duration at 20% relaxation (CD20), shortening time ($+C_{max}$) and relaxation time ($-C_{max}$) were not significantly different in the two cell groups.

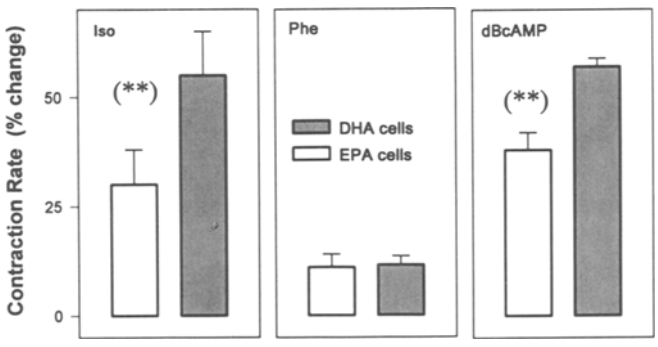


FIG. 2. Chronotropic influence of isoproterenol (Iso, 10^{-7} M), phenylephrine (Phe, 3×10^{-6} M), and dibutyryl AMP (dBcAMP, 10^{-3} M) in EPA and DHA cells. (**), $P < 0.01$; abbreviations as in Figure 1.

TABLE 1
Action Potentials (A) and Rate and Contraction Parameters (B) from Cultured Rat Cardiac Myocytes Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) Cells[a]

A	MDP (–mV)	OS (mV)	APA (mV)	APD80 (ms)	V_{max} (V/s)
DHA cells	79 ± 1.2	15 ± 0.6 —[b]	95 ± 1.2 —[b]	139 ± 7.1	67 ± 10.0
EPA cells	81 ± 0.9	17 ± 0.9	98 ± 1.3	135 ± 3.9	73 ± 11.3
B	F (m^{-1})	CD20 (ms)	CD80 (ms)	$+C_{max}$ (ms)	$-C_{max}$ (ms)
DHA cells	269 ± 17.4	64 ± 4.7	156 ± 9.1	42 ± 1.8	51 ± 2.6
EPA cells	257 ± 15.3	69 ± 3.5	167 ± 6.8	45 ± 1.4	54 ± 2.2

[a]Values are the mean ± SEM (n = 10). Abbreviations: MDP, maximal diastolic potential; OS, overshoot; APA, action potential amplitude; APD80, action potential durations at 80% repolarization; V_{max}, maximal rate of initial depolarization of the action potential; F, spontaneous rate; CD20, CD80, contraction duration at 20 and 80% relaxation, respectively; +Cmax, –Cmax, shortening and relaxation time (measured between 20 and 80% full contraction amplitude), respectively.
[b]$P < 0.05$.

α-Adrenergic function. Both DHA and EPA cells reacted to phenylephrine addition (3×10^{-6} M), which provoked an increase in spontaneous beating frequency (Fig. 2). These chronotropic responses were of a small magnitude and represented a 10% increase in rate. The positive chronotropic effect was similar in DHA- and EPA-treated cells (the average rate elevation was 17 and 16 beats/min, respectively). The phenylephrine-induced contraction shortening in DHA cells was not significantly different from that observed in EPA cells. The density of α1 receptor binding sites expressed by β_{max} were quite similar in DHA- and EPA-treated cells (36 ± 2.6 and 37 ± 3.9 fmoles, respectively). Similarly, the affinity of these sites for [^{3}H]-prazosin expressed as Kd was not significantly different in DHA and EPA cells (140 ± 21 and 115 ± 12 pM, respectively). In the two groups of cardiomyocytes, exposure to phenylephrine (3×10^{-6} M, 10^{-4} M) caused a highly significant, dose-dependent increase in [^{3}H]-inositol phosphates (Fig. 3). However, at each phenylephrine concentration considered, the [^{3}H]-inositol phosphate production was not significantly different in DHA and EPA cells.

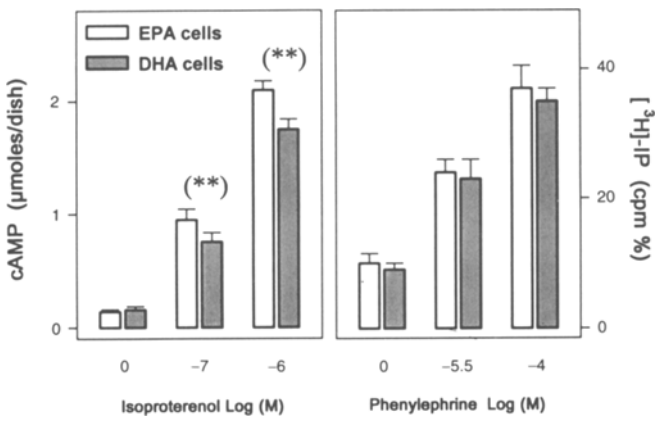


FIG. 3. Production of second messengers in EPA and DHA cells elicited by α- and β-adrenergic agonists (Phe and Iso, respectively). cAMP is expressed as µmole per dish and [^{3}H]-inositide phosphates as % of phosphatidylinositol radioactivity. (**), $P < 0.01$; abbreviations as in Figures 1 and 2; [^{3}H]-IP, [^{3}H]-inositide phosphates.

β-Adrenergic function. In the two groups, the addition of isoproterenol (10^{-6} M) caused an increase in spontaneous frequency of beatings, much more pronounced than that elicited by phenylephrine. However, the chronotropic response was significantly higher in DHA cells relative to EPA cells (Fig. 2). Additionally, isoproterenol induced a shortening of the contraction duration (data not shown), and DHA and EPA cells seemed to differ in this respect, as the isoproterenol-induced contraction shortening was significantly more pronounced in the DHA-rich cardiomyocytes. However, because contraction duration is a rate-dependent parameter, the observed difference resulted from the effect of DHA on the rate. The density of β-adrenergic receptor binding sites, as evaluated by β_{max} determination, was similar in DHA and EPA cells (42 ± 4.5 and 40 ± 3.5 fmoles, respectively). Conversely, the affinity of the β-adrenergic receptors for dihydroalprenolol tended to be lowered by DHA enrichment, as the Kd was higher in DHA- as compared to EPA-treated cells (400 ± 15 and 330 ± 28 pM, respectively). The production of cAMP in EPA and DHA cells is presented in Figure 3. In the absence of external β-adrenergic stimulation, the intracellular content in cAMP was similar in the two groups: 155 ± 16.0 nmoles/dish in DHA cells vs. 140 ± 10.8 nmoles/dish in EPA cells. The amounts of cAMP produced were strongly enhanced by isoproterenol stimulation in both groups, but the rise in cAMP induced by isoproterenol was significantly lower in DHA cells than in EPA cells (Fig. 3).

DISCUSSION

The results presented here indicate that increasing the DHA content resulted in a depressed plateau potential level, which is governed by the calcium ion channels. A relationship between EPA supplementation and calcium homeostasis has been suggested (11), but comparison with DHA was not considered. Increasing the n-3 PUFA content in PL was also reported to alter calcium transport in isolated cardiac myocyte

(9), but the study involved a change in n-6/n-3 ratio and an increase in both EPA and DHA. In a previous work, we showed that a modification of the n-6/n-3 ratio elicited by C_{18} precursors did not influence the action potential. All these results contribute to the hypothesis that some calcium channels may be influenced by changes in the fatty acid composition of the membrane, provided that these changes concern the DHA level. Similarly, the nature of the long-chain n-3 PUFA (EPA vs. DHA) in the ventricular myocyte PL do influence other membrane functions, such as the phospholipase A_2 activity (23), but not the spontaneous rate expressed by cardiomyocytes in culture, in spite of the fact that external calcium at least partly controls the beating rate of the cultured rat heart cells (32). This study confirms that membrane PUFA have no chronotropic influence in isolated cardiomyocytes (12). This is consistent with the observation that chronic EPA administration has no significant effect on heart rate in SHR and WKY rats (33). Additionally, increasing the DHA content without major alteration of the n-6/n-3 ratio did not affect the resting diastolic potential, the upstroke velocity, and the depolarization configuration. According to Karmazyn *et al.* (9), increasing both EPA and DHA and decreasing n-6 PUFA had no effect on the resting potential of rat cardiac myocytes, but delayed the final repolarization phase. The contraction duration and the shortening and relengthening times in ventricular myocytes were not affected by the DHA content, nor were they affected by the n-6/n-3 PUFA ratio (12). According Hallaq *et al.* (11), the amplitude of cell contraction is not altered by EPA incorporation, and the contraction was reported to be independent from DHA content in cardiac myocyte (34). Although myocardial contractility seems to be sensitive to an increase in n-6 PUFA (35), marked changes in the PUFA profile of heart cell membranes do not appear to affect myocardial performances (36–38). Recently, Finkel *et al.* (39) reported that EPA caused negative inotropic response in isolated papillary muscle, but the extent of fatty acid alteration was not characterized.

In a previous paper, we reported that increasing the n-3 PUFA in membrane PL enhanced the chronotropic response to isoproterenol (27). The present study provided the additional data that this chronotropic response was strengthened when EPA and DPA were replaced by DHA in the PL, although the n-6 vs. n-3 ratio was the same. Therefore, the individual PUFA may exert different effects on the myocardial β-adrenergic system. The results of this study suggest that the amount of DHA incorporated in cardiac membranes following fish oil (or n-3 PUFA) diets should be considered as a major factor, and not only the total n-3 incorporation. The DHA vs. EPA + DPA balance did not change the β-receptor binding site number (β_{max}), whereas a higher DHA level in the PL tended to induce a slight decrease in the affinity for dihydroalprenolol. Similar alterations were already reported in the heart from fish oil-fed rats (15). The balance between DHA and arachidonic acid in the membrane PL may modulate the age-dependent changes in the number and the affinity of β1-receptors (8). However, the increase in DHA decreased the isoproterenol-stimulated accumulation of cAMP. Numerous papers suggested the sensitivity of cardiac adenylate cyclase to membrane lipid environment, but often led to discrepancies (13,40). Laustiola *et al.* (41) reported that fish oil fed rats displayed a low isoproterenol-stimulated cAMP accumulation. Conversely, an enhanced catecholamine-stimulated adenylate cyclase activity has been observed in fish oil-fed pigs and rats (33,40). Again, the adenylate cyclase activity and its coupling to β-receptors may depend on the actual DHA amount in the PL as much as on the n-6 vs. n-3 balance. To further explore the apparent opposite effects of DHA on β-chronotropy and cAMP production, we studied the specific responses to dBcAMP, a permanent cAMP analogue. dBcAMP induced a significantly higher rise in rate in DHA-rich cells. The specific influence of membrane DHA content on the β-adrenergic chronotropy appears to result partly from an enhanced functional efficiency of cAMP. Membrane DHA could influence the regulation of membrane-bound components linked to automaticity, but also to the intracellular steps of the signal transduction system, such as protein kinases and/or phosphodiesterase (40). However, an influence of DHA on phosphodiesterase activity seems doubtful, as the dibutyryl derivative of cAMP used in this study is known to be phosphodiesterase-resistant. The present study provided the additional data that the DHA level in the cardiomyocyte PL did not influence their positive chronotropic response to phenylephrine, nor the rate of inositol phosphate formation, nor the density and affinity of α1-binding sites. n-3 PUFA were reported to reduce the α-adrenoceptor mediated contractile response in the isolated heart and artery (18,42). Recently, the modifications in membrane PL achieved by hormonal treatment was also shown to affect the α1-adrenergic receptor affinity, but this effect could not be related to specific fatty acid membrane changes. Accordingly, Meij *et al.* (43) reported that PUFA composition of membrane PL has no effect on the total amount of α1-stimulated inositol phosphates produced in rat heart cell cultures. However, Lamers *et al.* (44) showed that a treatment of ventricular myocytes with either linoleic acid or EPA reduced the rate of inositol phosphate production. In this study, the n-3 PUFA composition in the phosphatidylinositol fraction was markedly altered, but not the arachidonic acid content (data not shown). This suggests that the affinity of phospholipase C for the phosphatidylinositol (and, additionally, the composition of the diacylglycerol messenger) do not depend on the n-3 composition, provided that the arachidonic acid content is not altered.

In conclusion, this work provided evidence for a differential effect of the two major fish oil PUFA. Increasing the DHA content in cardiomyocyte membranes did not modify the function of the α-adrenergic system, in spite of the known involvement of PL in the α-adrenergic mechanism. Conversely, a high DHA content in the PL depressed the β-adrenergic induced cAMP production, but improved the functional efficiency of cAMP, which resulted in an enhanced chronotropic response to a β-agonist.

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Quantification of Distinct Molecular Species of Platelet Activating Factor in Ulcerative Colitis

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ABSTRACT: The aim of this study was to characterize the synthesis and metabolism of platelet activing factor (PAF, 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) by colonic mucosa from patients with ulcerative colitis and healthy individuals. Tissue was obtained by endoscopic biopsy and by scraping the mucosa from surgical resections. Tissue was assayed for the various molecular species of PAF and its biologically inactive metabolite lyso-PAF using gas chromatography/mass spectrometry. Mucosa from surgical resections for ulcerative colitis contained C16:0 PAF (mean = 156 ng/g of mucosa), but not C18:0 PAF. PAF was not identified in mucosa from normal surgical resections or in endoscopic biopsies from either patients with ulcerative colitis or normal individuals. Both C16:0 lyso-PAF and C18:0 lyso-PAF were found in mucosa from normal and ulcerative colitis surgical resections and in endoscopic biopsies from ulcerative colitis and normal tissue. Levels of lyso-PAF were similar in ulcerative colitis and normal mucosa. Incubation of mucosa from areas of active inflammation in ulcerative colitis with the calcium ionophore A23187 increased the levels of C16:0 PAF by 2–3 fold and also increased the levels of C16:0 lyso-PAF. Addition of ^{3}H-PAF to endoscopic biopsies from either normal individuals or patients with ulcerative colitis resulted in hydrolysis to ^{3}H-lyso-PAF. The data on colonic mucosal levels of PAF are consistent with the results of earlier studies measuring PAF in patients with ulcerative colitis by bioassay. This study examines the synthesis and metabolism of specific molecular species of PAF in ulcerative colitis for the first time.
Lipids 31, S-255–S-259 (1996).

Platelet activating factor (PAF, 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent mediator of inflammation that causes neutrophil activation, increased vascular permeability, and vasodilation in addition to platelet activation (1). Increased levels of PAF have been found in inflamed mucosa from patients with ulcerative colitis and Crohn's disease and in the stools of patients with Crohn's disease and pouchitis after colectomy for ulcerative colitis (2–6). PAF is also thought to play an important role in the pathogenesis of necrotizing entercolitis (7). Inflammatory cells, particularly mast cells and tissue macrophages, and gastrointestinal epithelial cells have been identified as cellular sources for PAF (8). Drugs used in the treatment of inflammatory bowel disease (including corticosteroids and 5-ASA compounds) inhibit the production of PAF, raising the possibility that PAF may play an important pathogenic role in inflammatory bowel disease (2,9). In previous studies in inflammatory bowel disease, PAF has been measured by bioassay using platelet aggregation in an aggregometer (2–6). Although platelet aggregation is a highly sensitive assay for PAF, its interpretation is confounded by the interference of other products in biological samples that might enhance (ADP, certain prostanoids) or diminish (adenosine, endogenous inhibitors) platelet aggregation (10). Moreover, there are several molecular species of PAF that have biologic activity, and the platelet aggregation assay does not allow the separation of the contribution of the various molecular species.

In this study, a stable isotope dilution gas chromatographic-mass spectrometric assay (11) was applied to characterize the levels of the various molecular species of PAF and its biologically inactive metabolite, lyso-PAF, in normal human colonic mucosa and in histologically inflamed mucosa from patients with ulcerative colitis.

METHODS

Tissue collection. Endoscopic biopsies were obtained during routine colonoscopies. Three to ten endoscopic biopsies were obtained from endoscopically inflamed colons from patients with active ulcerative colitis and from endoscopically normal areas from healthy individuals undergoing screening colonoscopy. Each endoscopic biopsy weighed approximately 10 mg. Endoscopic biopsies include epithelial cells and some lamina propria inflammatory cells. Endoscopic biopsies were obtained from inflamed colonic mucosa in 11 patients with active ulcerative colitis (6 men and 5 women, mean age 41 years with a range of 22–78 years) and 8 healthy individuals (4 men and 4 women, mean age 56 years with a range of 33–85 years). Material from surgical resections was collected from the operating room. Surgical resections came from patients undergoing total colectomies for active ulcerative

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Abbreviations: GC, gas chromatography; PAF, platelet activating factor; PBS, phosphate-buffered saline; PFB, pentafluorobenzoyl; TLC, thin-layer chromatography.

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colitis resistant to medical therapy (3 men and 1 woman, mean age 34 years with a range of 21–48 years). Normal colonic tissue was obtained from the uninvolved areas of surgical resections for colon cancer (2 men and 1 woman, mean age 66 with a range of 60–72 years). The surgical resections were washed and mucosa was obtained by scraping the surgical resections with a glass slide. This method yields a preparation that includes the epithelium, the lamina propria, the muscularis mucosal and the submucosa down to the level of the muscularis propria.

PAF quantitation. The samples were placed in PBS (1 mL) and an internal standard d_3C16:0 PAF (10 ng) was added. The samples were extracted for total lipids by the method of Bligh and Dyer. For quantitation of any lyso-PAF in the samples, the lipid extract was treated with propionylchloride to convert lyso-PAF to propinoyl PAF (11). The extract was concentrated to dryness under N_2 and derivatized with 100 µL propionic acid and 50 µL propionyl chloride for 45 min at 60°C. This material was dried (N_2) and reconstituted in 1 mL chloroform. PAF molecular species were purified on silicic acid extraction columns (Sep-Pak; Fischer Scientific, Fairlawn, NJ). Columns were conditioned with 5 mL ethyl acetate and 5 mL methanol using negative pressure to draw the solvent through the columns. The samples were applied to the columns and then washed with 5 mL of 1% acetic acid in chloroform and then with 5 mL of chloroform/methanol, 1:1, vol/vol. PAF was then eluted from the columns with 5 mL chloroform/methanol/water, 1:2:0.8, by vol. Chloroform and water (1.3 mL) were added to the column eluate to effect phase separation and the chloroform phase was collected and concentrated to dryness. This purified material was converted to the analogous diglyceride by digestion with phospholipase C (from *B. cereus;* Sigma, St. Louis, MO) (25 units phospholipase C in 250 µL 0.2 M sodium borate buffer, pH 8.0 with 2 mM Ca^{2+}). One mL of diethyl ether was added, and the samples were tightly capped and incubated in a shaking 37°C water bath for 90 min. The ether layer was subsequently removed, dried, and the resulting diglyceride was converted to the pentafluorobenzoyl (PFB) derivative with 10 µL pentafluorobenzoyl chloride (Sigma) in 50 µL of 4% pyridine in toluene at 80°C for 90 min. After concentrating to dryness, the PAF-PFB derivative was purified by thin-layer chromatography (TLC) on silica gel-G plates (Analtech, Newark, DE). Plates were developed first in hexane, allowed to dry, and then developed in hexane/ethyl acetate, 9:1, vol/vol. The derivatives of PAF and lyso-PAF were eluted from the silicic acid with hexane:ethyl acetate, 9:1, vol/vol. Migration positions were determined with authentic PAF-PFB standards run on outer lanes of the TLC plate. The hexane/ethyl acetate solutions containing the recovered PAF and lyso-PAF derivatives were back extracted twice with water and concentrated to dryness. Samples were then reconstituted to 20 µL heptane and analyzed by capillary column gas chromatography-negative ion chemical ionization mass spectrometry.

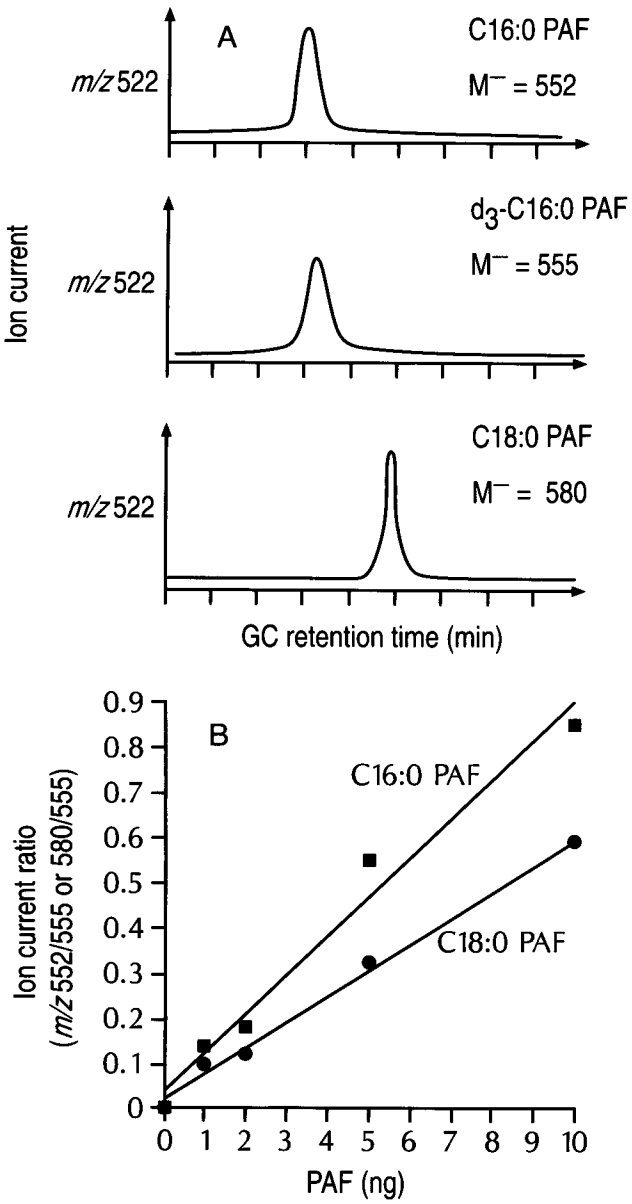


FIG. 1. Gas chromatography (GC)-mass spectrometry analysis of platelet activating factor (PAF) derivatives. The left panel shows the ion currents products by the derivatized diglycerides of C16:0 PAF, the d_3-C 16:0 PAF internal standard, and C18:0 PAF. The right panel shows typical standard curves demonstrating the correlation of authentic PAF vs. a fixed amount of the internal standard expressed as the ratio of their respective ion currents: *m/z* 552/555 for C16:0 PAF, and *m/z* 580/555 for C18:0 PAF.

Gas chromatography-mass spectrometry. Gas chromatography (GC) was performed on a Hewlett Packard 5890 gas chromatograph with a fused silica capillary column 8m Hewlett Packard Ultraperformance (methysilicone, 0.17 micron film thickness, 0.32 mm i.d.) interfaced with a Hewlett Packard model 5988 quadrapole mass spectrometer (11). Injections were made in the splitless mode with helium as the carrier gas (total flow 10 mL/min, head pressure 4 psi). The GC oven temperature was programmed from 85 to 300°C at 40°C per min beginning 0.8 min after injection. Temperatures of the GC interface and injector were maintained at 285°C. Mass spectrometry was performed in the negative ion chemical ionization mode with methane as the reactant gas (source pressure 1.5 torr). Selective ion monitoring of the following ions was performed *m/z*: 552 (C16:0-

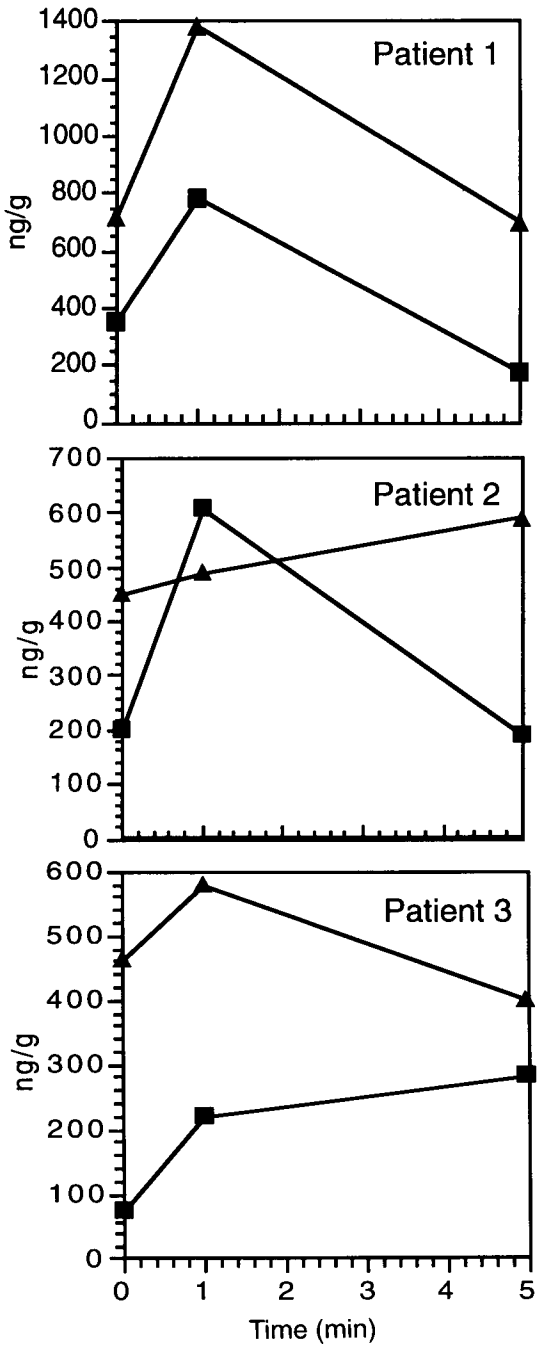


FIG. 2. Production of C16:0 PAF and C16:0 lyso-PAF by mucosa scraped from ulcerative colitis surgical resections (30 mg) incubated with A23187 (1 μM) for 0, 1, and 5 min. The tissue and media were extracted together and molecular species of PAF and lyso-PAF were measured by gas chromatography-mass spectrometry. Each panel represents a separate patient. Abbreviation as in Figure 1.

PAF); 566 (2-propionyl derivative of lyso-C16:0-PAF derivative); 580 (C18:0-PAF); 578 (C18:1-PAF); and 555 ($^2H_3$-labeled C16:0-PAF internal standard). Standard curves for PAF-PFB and lyso-PAF-PFB-propionyl derivatives were performed with each assay by combining various concentrations of the appropriate PAF or lyso-PAF standards with 10 ng of d_3C16:0 PAF internal standard. Each standard was subjected to the same isolation, purification, and derivatization procedures described above for the samples obtained from the allograft tissue. Representative gas chromatography-mass spectrometric tracings and a standard curve obtained from one experiment are shown in Figure 1.

Incubation with A23187. Samples of scraped colonic mucosa (20–40 mg), or colonoscopic biopsies (20–40 mg) were incubated with A21387 (1 μM) at 37° in phosphate-buffered saline (PBS) for various times. The tissue samples and media were extracted together by the method of Bligh and Dyer and processed for PAF and lyso-PAF quantitation as described above.

Degradation of ^{3}H-PAF. Three endoscopically obtained mucosal biopsies from either normal subjects or patients with ulcerative colitis were placed in Dulbecco's modified Eagle's medium with 1-O-^{3}H-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (8.3 nM) and incubated for various periods at 37°C. The tissue and media were extracted by the method of Bligh and Dyer, and the lipids were separated by TLC on silica gel-G. The solvent system used was chloroform/methanol/acidic acid/water (50:25:8:4, by vol). Each lipid class was identified on the basis of comigration with standards and the corresponding areas were scraped and radioactivity assessed in a Beckman scintillation counter.

RESULTS

A stable isotope dilution gas chromatographic-mass spectrometric assay was used to measure the levels of various molecular species of PAF and lyso-PAF in histologically normal human colonic mucosa and in histologically inflamed mucosa from patients with ulcerative colitis. Detectable amounts of C16:0 PAF (156 ± 141 ng/g) were found in mucosa scraped from four of four surgical resections for active ulcerative colitis (Table 1). No detectable levels of C16:0 PAF were found in ulcerative colitis endoscopic biopsies or in mucosa scraped from histologically normal endoscopic biopsies. No detectable C18:0 PAF was found in any of the surgical resections or endoscopic biopsies from either ulcerative colitis patients or normals. The lower limit of sensitivity of the gas chromatography-mass spectrometry assay for C16:0 PAF or C18:0 PAF is 1 ng (Fig. 1); with 30 mg tissue samples the lower limit of sensitivity is 33 ng/g of mucosa. Detectable levels of C16:0-lyso-PAF and C18:0 lyso-PAF were found in all of the surgical resections and all of the endoscopic biopsies. There were no significant differences in the levels in the two molecular species of lyso-PAF between samples taken

TABLE 1
Molecular Species of PAF and Lyso-PAF in Ulcerative Colitis (UC) and Histologically Normal Colonic Mucosa

	C16:0-PAF (ng/g)	C18:0-PAF (ng/g)	C16:0-lyso-PAF (ng/g)	C18:0-lyso-PAF (ng/g)
UC resections (n = 4)	156 + 71[a]	< 33	390 ± 118	363 ± 200
UC biopsies (n = 11)	< 33	< 33	622 ± 133	414 ± 145
Normal resections (n = 3)	< 33	< 33	510 ± 170	634 ± 479
Normal biopsies (n = 8)	< 33	< 33	639 ± 85	441 ± 117

[a]Mean ± SEM.

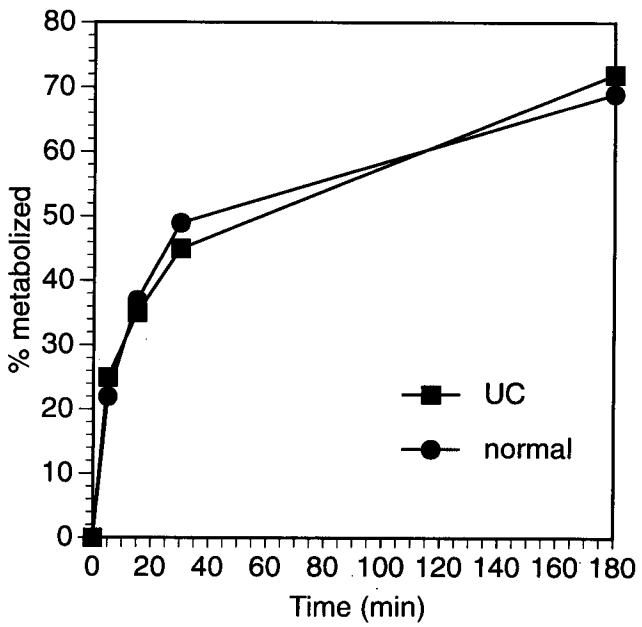


FIG. 3. Metabolism of ^{3}H-C16:0 PAF by mucosal biopsies from normals or patients with ulcerative colitis. Three endoscopically obtained mucosal biopsies from patients with ulcerative colitis were placed in Dulbeco's modified Eagle medium with 1-O-^{3}H-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (8.3 nM) and incubated for various times at 37°C. The tissue and media were extracted by the method of Bligh and Dyer and the lipids were separated by thin-layer chromatography. The data are presented as the percent of added ^{3}H-PAF metabolized at each time point. Abbreviation as in Figure 1.

from ulcerative colitis and normal individuals. There were also no significant differences in lyso-PAF levels between endoscopic biopsies and surgical resections. In contrast to PAF, where only the C16:0 molecular species was found, the C16:0 and C18:0 molecular species of lyso-PAF were present in roughly equivalent amounts.

To test the ability of colonic mucosa to synthesize PAF, surgical resections and endoscopic biopsies were incubated with the calcium ionophore A23187. The production of PAF and lyso-PAF species was measured after incubations with A23187 for 1 and 5 min. After incubation with A23187, C18:0 PAF was not detected in any sample. After incubation with A23187, C16:0 PAF production was observed only in those samples which had contained measurable basal amounts of C16:0 PAF prior to the addition of A23187. Incubation of mucosa from three of the four ulcerative colitis surgical resections with A23187 resulted in synthesis of C16:0 PAF (Fig. 2). PAF synthesis in the fourth specimen was not studied. Two of the three samples demonstrated 2–3 fold increases in PAF levels after 1 min incubation in the A23187 with a decline at 5 min. In the third sample, there was a 2-fold increase in PAF levels at 1 min and a 3-fold increase at 5 min. In each case, the increase in 16:0 PAF was accompanied by an increase in 16:0 lyso-PAF. This is consistent with degradation of PAF by acetylhydrolase.

The typical pathway for PAF degradation is hydrolysis of the acetyl group to yield acetate and lyso-PAF. Finding that lyso-PAF levels were consistently higher than PAF levels in both normal mucosal and inflammatory bowel disease mucosa raised the possibility that PAF is rapidly degraded in the human intestinal mucosa. An experiment was performed to determine whether PAF is degraded in human colonic mucosa and to assess whether there is a difference in the rate of PAF degradation between normal and ulcerative colitis mucosa. ^{3}H-PAF was incubated with colonoscopic biopsies from normal and ulcerative colitis tissue. After various times, the incubations were terminated. Lipids were then extracted from the tissues and the media together, and the radiolabelled metabolites analyzed by thin-layer chromatography. PAF was metabolized to lyso-PAF, which was further metabolized to phosphorylcholine and alkylglycerols. The rate of PAF metabolism by endoscopic biopsies from histologically normal tissue and ulcerative colitis tissue was equivalent. In both systems, 50% of the added ^{3}H-PAF was metabolized within 30 min. (Fig. 3).

DISCUSSION

In this study, we detected C16:0 PAF in inflamed colonic mucosa from patients with active ulcerative colitis, but not from histologically normal tissue. C18:0 PAF was not detected in either ulcerative colitis tissue or histologically normal colonic tissue. Both C16:0 lyso-PAF and C18:0 lyso-PAF were found in biopsies and mucosal scrapings from normal and ulcerative colitis colonic mucosa. There were no significant differences in lyso-PAF content of normal and diseased mucosa.

In earlier studies, in which PAF activity was measured by platelet aggregation, detectable quantities of PAF were found in biopsies from patients with ulcerative colitis but not in biopsies from normal individuals (2). In our study in which PAF molecular species were measured by gas chromatography-mass spectrometry, detectable levels of C16:0 PAF were found in mucosal scrapings from surgical resections for ulcerative colitis but not in ulcerative colitis biopsies. There are several factors which may contribute to these apparent differences. Probably the most important is that the gas chromatography-mass spectrometry assay, although more quantitative and specific, is less sensitive than platelet aggregation bioassay. Using 30 mg tissue samples, the lower limit of sensitivity of the gas chromatography-mass spectrometry assay is 33 ng of PAF per gram of mucosa. In platelet aggregation assays, aggregation occurs in response to as little as 5 picograms of synthetic PAF. Using the platelet aggregation assay PAF levels as low as 1 ng/g were measured in ulcerative colitis endoscopic biopsies (2). The differences between measurements of PAF levels in ulcerative colitis endoscopic biopsies in the study described here and earlier studies can therefore be explained by differences in the sensitivity of the assays. The ability of the gas chromatography-mass spectrometry method to detect C16:0 PAF in mucosal scrapings from ulcerative colitis surgical resections but not from endoscopic biopsies may reflect the higher levels of PAF in the surgical resections due to the higher level of disease activity in patients undergoing surgical resections for ulcerative colitis.

The two major biologically active molecular species of PAF are the C16:0 and C18:0 species. Relatively few studies have measured the distinct molecular species in biological samples. Where they have been measured, the C16:0 PAF has been quantitatively more significant than C18:0 PAF (12,13). For example, in transplanted dog kidneys undergoing rejection, 16:0 PAF was present in the cortex at levels 80-fold greater than C18:0 PAF and in the medulla at levels 20-fold greater (12). Our findings of high levels of 16:0 PAF and undetectably low levels of 18:0 PAF are consistent with these earlier observations. The C16:0 PAF levels in ulcerative colitis mucosal scrapings measured in this study by gas chromatography-mass spectrometry are somewhat higher than PAF levels reported previously in endoscopic biopsies from ulcerative colitis mucosa reported previously using platelet aggregation. This difference may reflect the greater level of disease activity in patients undergoing surgical resections. Although this study did not address the cellular source of the C16:0 PAF in ulcerative colitis mucosa, the results of an earlier study suggest that both inflammatory cells and epithelial cells may contribute (8).

This is the first study to measure lyso-PAF by chemical methods in inflammatory bowel disease and the first to investigate the molecular species of lyso-PAF. Earlier studies had measured lyso-PAF by converting lyso-PAF to PAF by treatment with acetic anhydride and then measuring PAF activity by platelet aggregation (4). Measurement of lyso-PAF by gas chromatography-mass spectrometry is more direct, more quantitative, and allows the separation of distinct molecular species. The finding that lyso-PAF was similar in ulcerative colitis tissue and normal tissue was not surprising. Lyso-PAF is generated by processes in addition to the hydrolysis of PAF. Lyso-PAF is produced from PAF by the action of acetylhydrolase. It can also be produced from plasmanycholine by the action of phospholipase A_2. Lyso-PAF levels are also regulated by the rate of reacylation to form plasmanylcholine and by the rates of degradation by phospholipases C and D to form diglycerides and lysoplasmanic acid. Although PAF levels are higher in ulcerative colitis colonic mucosa than normal colonic mucosa there is no evidence that the other determinants of lyso-PAF levels are different in ulcerative colitis and normal colon.

Increased PAF production in ulcerative colitis fits with enhanced production of other mediators. The first step in the synthesis of PAF through the remodeling pathway is the hydrolysis of 1-alkyl-2-acyl-*sn*-glycero-3-phosphcholine by phospholipase A_2. In addition to releasing lyso-PAF, this reaction also releases arachidonic acid, the substrate for the synthesis of prostaglandin E_2 and leukotriene B_4, both of which are present at increased levels in ulcerative colitis (14).

In summary, in this study a stable isotope dilution gas chromatographic-mass spectrometric technique was used to measure molecular species of PAF and lyso-PAF in histologically normal colonic mucosa and in inflamed mucosa from patients with ulcerative colitis. The inflamed mucosa contained detectable amounts of C16:0 PAF but not C18:0 PAF. Normal mucosa did not contain detectable amounts of either molecular species of PAF. Significant amounts of C16:0 lyso-PAF and C18:0 lyso-PAF were found in all samples with no significant difference between the levels in the normal mucosa and ulcerative colitis mucosa.

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Skeletal Muscle Membrane Lipids and Insulin Resistance

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ABSTRACT: Skeletal muscle plays a major role in insulin-stimulated glucose disposal. This paper reviews the range of evidence in humans and experimental animals demonstrating close associations between insulin action and two major aspects of muscle morphology: fatty acid composition of the major structural lipid (phospholipid) in muscle cell membranes and relative proportions of major muscle fiber types. Work *in vitro* and *in vivo* in both rats and humans has shown that incorporation of more unsaturated fatty acids into muscle membrane phospholipid is associated with improved insulin action. As the corollary, a higher proportion of saturated fats is linked to impairment of insulin action (insulin resistance). Studies *in vitro* suggest a causal relationship. Among polyunsaturated fatty acids (PUFA) there is some, but not conclusive, evidence that ω-3 (n-3) PUFA may play a particular role in improving insulin action; certainly a high n-6/n-3 ratio appears deleterious. In relation to fiber type, the more highly oxidative, insulin-sensitive type 1 and type 2a fibers have a higher percentage of unsaturated fatty acids, particularly n-3, in their membrane phospholipid, compared to the insulin-resistant, glycolytic, type 2b fibers. These variables, however, can be separated and may act in synergy to modulate insulin action. It remains to establish whether lifestyle (e.g., dietary fatty acid profile and physical activity), genetic predisposition, or a combination are the prime determinants of muscle morphology (particularly membrane lipid profile) and hence insulin action.
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Skeletal muscle is the major site of insulin-stimulated glucose disposal. Insulin resistance, relative failure of insulin action, and associated hyperinsulinemia are strongly linked to the development of a cluster of prevalent diseases, including noninsulin-dependent diabetes mellitus (NIDDM), obesity, hyperlipidemias, hypertension, and heart disease (1–3). While skeletal muscle insulin resistance has clearly been demonstrated in both human and experimental animal models of obesity and diabetes, the mechanisms are still poorly understood. Work from our laboratory, and others, has focused on interrelationships between fatty acid composition of muscle membrane structural lipids (phospholipids), levels of muscle storage triglyceride, muscle fiber composition, and insulin resistance. This short review is basically limited to studies in skeletal muscle and concentrates on work from our laboratory.

Muscle phospholipid composition and insulin action. A number of lines of evidence led to the current work. First, it was known from the pioneering work of Felber and colleagues (4) that diets high in fat led to increased accumulation of storage triglyceride in skeletal muscle. According to the glucose–fatty acid cycle of Randle and coworkers (5,6) and inhibitory effects of lipids on glucose storage (7), this lipid accumulation was a plausible factor in impairment of muscle glucose metabolism. The second important set of observations were noted when the euglycemic clamp for measurement of insulin action was adapted for use in rats (8,9), and the technique was further extended to use of the labeled 2-deoxyglucose method for assessing insulin action in individual tissues (10,11). Using these techniques, it was possible to demonstrate directly that high-fat feeding induced insulin resistance in individual skeletal muscles (12,13). Further, by combining ^{3}H-labeled 2-deoxyglucose and ^{14}C-labeled glucose bolus injection during the euglycemic clamp, it was possible to show that fat-feeding induced insulin resistance in both the storage and oxidation/glycolysis components of muscle glucose metabolism.

The third line of evidence came from work (14) showing hypotriglyceridemic properties of n-3 fatty acids. The question became, if high-fat diets lead to muscle insulin resistance via the accumulation of storage lipid, would inclusion of lipid-lowering n-3 fatty acids in the high-fat diet be beneficial? Indeed that was the observation. When n-3 fatty acids were substituted into a high-fat diet, insulin resistance in skeletal muscle was prevented (15). Importantly, both the storage and oxidation/glycolysis components of glucose metabolism were ameliorated by inclusion of the n-3. The observation that both components of glucose metabolism in muscle were impaired by some high-fat diets, and both ameliorated by n-3 fatty acids, was suggestive of either multiple metabolic defects or a single defect at an early, common point in the glucose metabolic pathway, perhaps at the membrane level.

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Abbreviations: FABP, fatty acid binding protein; NIDDM, noninsulin-dependent diabetes mellitus; PUFA, polyunsaturated fatty acids; UI, unsaturation index.

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This seminal observation of the profound dependency of high-fat diet–induced insulin resistance *in vivo* on the fatty acid profile of the diet was consistent with number of *in vitro* studies. These investigations have provided evidence that changes in the composition of fatty acids within membrane phospholipids strongly influence insulin action, altering both insulin binding and action. In general, the more saturated the fatty acids in membrane phospholipid, the more deleterious the effect (16,17). Further, the highly unsaturated n-3 fatty acids may be particularly beneficial (18,19). Our recent studies using L6 myocytes have extended these observations to a muscle-derived cell line (Pan and Storlien, unpublished).

Follow-up work was aimed at extending both *in vivo* and *in vitro* work in terms of elucidating mechanisms relating muscle membrane lipid profile and insulin action. An extensive study was carried out in rodents comparing a number of high-fat diets pair-fed and differing only in the fatty acid profile of the fat components (20). Results demonstrated marked intergroup differences in insulin-stimulated glucose metabolism when assessed using the euglycemic clamp. Some high-fat–fed groups progressed to major insulin resistance, and others did not, with effects particularly pronounced in skeletal muscle. As we had originally proposed, a variable closely associated with muscle insulin resistance was accumulation of storage lipid, consistent with the accepted glucose/lipid interactions. Further, since earlier results had suggested that the metabolic defect induced by some forms of high-fat feeding was at the membrane level, it was reasoned that changing the dietary fatty acid profile might also change the fatty acid profile of membrane structural lipid, and that, in turn, may influence insulin action. Indeed, improved insulin action was clearly associated with an increased percentage of longer carbon chain, more highly unsaturated fatty acids in the muscle membrane phospholipid.

The association between membrane lipid profile and insulin action in experimental animals prompted studies in humans. The first work assessed skeletal muscle phospholipid fatty acid composition in normoglycemic volunteers undergoing coronary bypass operations and from whom rectus abdominous muscle biopsies were obtained. Results were consistent with those observed in rodents. Fasting insulin level, used as an index of insulin resistance in this normoglycemic population, was inversely correlated with the percentage of long-chain polyunsaturated fatty acids (PUFA) and with the unsaturation index (UI: the number of double bonds per fatty acyl moiety $\times$ 100) in the muscle membrane phospholipid. That is, the more unsaturated the membrane lipid, the more insulin-sensitive the individual (21). A follow-up study in young, healthy subjects used the hyperinsulinemic/euglycemic clamp to assess insulin action directly, and percutaneous biopsies of the vastus lateralis were obtained. The same relationships were found in this normal group between muscle membrane phospholipid profile and insulin action (21).

Two other studies in humans, using very different study populations, further explored these relationships. Both studies used the euglycemic clamp to assess insulin action directly and analyzed vastus lateralis muscle obtained by percutaneous biopsy. Vessby and colleagues studied an older, male Swedish population (22). Relationships were again found between insulin action and muscle phospholipid fatty acid composition. Differences between Australian and Swedish results did occur, however. In the Swedish study, the highest correlation occurred with saturated fatty acids (particularly palmitic). That is, the higher the proportion of saturated fatty acids in the muscle membrane phospholipid, the more insulin-resistant the individual. In contrast to the Borkman *et al.* study (21), relationships with the highly unsaturated fatty acids were not significant.

The third study in this series of work was carried out in the diabetes- and obesity-prone Pima Indians (23). Again, results congruent with the two previous studies were found: the more unsaturated the phospholipids, the better the insulin action. Here both individual saturated and polyunsaturated fatty acids were significantly linked to insulin action.

An interesting aspect of this last study was that it confirmed and extended observations in the original study of strong relationships between insulin action and measures of activity of the Δ5-desaturase enzyme. It is clearly true that the UI of the average Western diet is much less (UI $\approx$ 80; Australian Dietary Survey, National Health and Medical Research Council, 1991) than the UI of skeletal muscle phospholipid (which averages approximately 170 in our studies). With the exception of certain marine oils, the major dietary fatty acids must all be substantially elongated and desaturated to be transformed into the fatty acids that our results have shown to be associated with insulin sensitivity. Elongase and desaturase enzymes are crucial rate-limiting elements in this transformation. Activity of these enzymes can be determined by product/precursor ratios (for example, the ratio of 20:4n-6 to 20:3n-6 gives an index of activity of the Δ5-desaturase enzyme). While not an optimal method for measuring enzyme activity, our results in humans, both in an Australian, largely Caucasian sample (21) and in Pima Indians (23), have shown remarkably strong relationships between reduced Δ5-desaturase activity (20:4n-6/20:3n-6) and insulin resistance.

The other aspect of the Δ5-desaturase enzyme is that it is common to both the n-6 and n-3 fatty acid families; as such, there is competition between n-6 and n-3 fatty acids as substrates for desaturation. Certainly it is true that n-3 fatty acids are elongated and desaturated to a greater extent (to 22-carbon 5 and 6 double-bond fatty acids) than is true for the n-6 pathway where arachidonic acid (20:4) is the normal endpoint. This may be significant at a time in human history when we have been undergoing the first major experiment of a high level of dietary intake of n-6 fatty acids. This has been an outcome, driven by the cardiovascular literature, of the move to increase polyunsaturated fat intake and reduce saturated fat intake. Polyunsaturated seed oils available over the last 30 years have been largely n-6 in makeup. We have analyzed both our Australian and Pima Indian data in terms of the relationship between the n-6/n-3 ratio, insulin action (as indexed by fasting insulin in these normoglycemic subjects),

and obesity. Figure 1 shows the results. Clearly, the higher the n-6/n-3 ratio, the worse the insulin action and the higher the relative weight level. Interestingly, Pima Indians have a much lower level (<40%) of n-3 fatty acids than the Australian, largely Caucasian, samples. In turn, Swedish subjects of Vessby had an n-3 percentage in skeletal muscle phospholipid that was much higher again than even the Australian group and over 3 times that of the Pimas. These observations presumably reflect at least in part dietary intake of n-3 fatty acids. There is also, however, very likely to be a genetic predisposition to incorporation, elongation, and desaturation of specific fatty acids. In dietary analysis of a limited number of subjects in the Borkman *et al.* (21) study, relative amounts of, for example n-3 fatty acids, did not relate at all to apparent dietary intake. It will be interesting to determine if low levels of n-3 fatty acids in Pima Indian muscle membranes relate at all to low dietary intake or reflect a genetic predisposition. It is interesting in this regard that an intestinal fatty acid binding protein (FABP) loci on chromosome 4q has been significantly linked with insulin resistance in this population (24). It is therefore possible that the "thrifty gene" (25,26) may include control, binding, and uptake of specific fatty acids and their insertion into membranes.

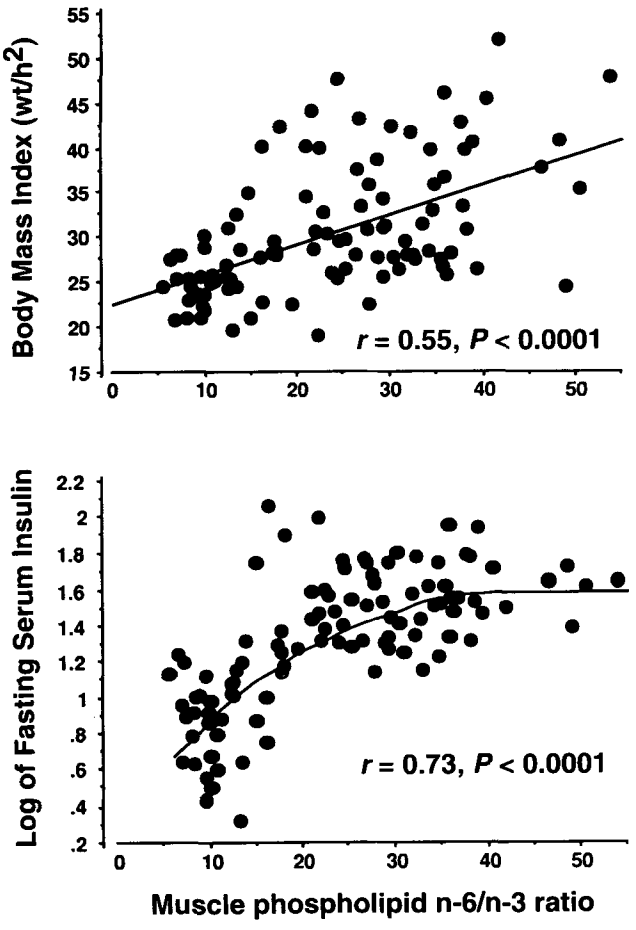


FIG. 1. Relationship between the n-6/n-3 ratio and relative adiposity (body mass index—top) and the n-6/n-3 ratio and insulin resistance (indexed by a high fasting serum insulin—bottom). The ratio of n-6/n-3 fatty acids was measured in phospholipids of muscle obtained by percutaneous biopsy of the vastus lateralis. Subjects mostly Caucasian Australians and Pima Indians (Storlien, L.H., Pan, D.A., and Lillioja, S., unpublished observations).

Relationships between muscle phospholipid fatty acid profile and insulin action have been established in adult rats and humans; however, the relative contributions of lifestyle and genetic predisposition are still unclear. In an effort to shed light on this issue, we have begun studies in human infants. Muscle biopsies are being obtained during surgery on infants who are not acutely unwell and who are near normal height and weight (operations range from talipes correction to minor gastric and cardiac repair). Initial results emphasize the major effect of diet. Breast-fed infants have a muscle membrane lipid profile much like insulin-sensitive adults. Formula-fed infants, in contrast, present a profile characteristic of insulin resistance (27). Insufficient evidence has yet been collected to assess the genetic predisposition to a certain lipid profile independent of the powerful dietary effect.

Relationship between membrane lipids and storage lipids. As noted earlier, the inhibitory effects of lipids on glucose metabolism have clearly been described (5–7). A significant feature of this glucose/lipid interaction appears to be the failure of insulin to suppress lipolysis of intramuscular storage triglyceride (28). We originally arrived at the relationship between dietary fat and insulin action via this issue. As noted earlier, a study with a large range of dietary fat profiles showed a good, inverse relationship between intramuscular triglyceride levels and insulin action in that muscle (20). In humans (29) we have now been able to show that intramuscular triglyceride levels are also closely related to whole-body insulin action independent of total adiposity. In this context it is interesting that we have recently demonstrated relationships between muscle membrane lipid composition and muscle storage lipid: the more saturated the membrane lipids, the greater the accumulation of storage lipid (30). This result is consistent with the observations of Leyton *et al.* (31), showing that more saturated fats are poorly oxidized for energy in comparison to PUFA and hence more likely to be stored (and incorporated into membranes). In addition, it is significant that, for a given carbon backbone number, n-3 fatty acids were markedly more likely to be quickly oxidized for energy than the equivalent n-6 fatty acid, which is more likely to be stored (see, for example, the 18-carbon n-6 and n-3 fatty acids).

Muscle fiber type, membrane lipids and insulin action. Finally, there are a number of aspects of muscle morphology that undoubtedly influence insulin action. One receiving current attention is the relative proportions of types 1, 2a, and 2b fibers. It has been known for some time that different fiber types exhibit enormously different sensitivity in relation to insulin's ability to promote glucose uptake (32). Type 1 (slow-twitch oxidative) and type 2a (fast-twitch oxidative/glycolytic) fibers are much more insulin-sensitive than type 2b (fast-twitch glycolytic) fibers (32). It has also been shown in humans that insulin resistance is associated with a relative in-

crease in percentage of type 2b fibers (33,34), a relative decrease in type 1 fibers (35), and indices of oxidative capacity (34,36).

To determine the relationship between muscle fiber type and membrane lipid composition, we have recently accumulated data in our laboratory on rats fed the same diet where the phospholipid fatty composition was determined on hindlimb muscles that were predominantly type 1 (soleus), type 2a (red quadriceps), or type 2b (white quadriceps). It was clear that the percentage of PUFA in membrane phospholipid was significantly higher in muscles that were predominantly of type 1 or type 2a fiber, as compared to muscles predominantly of type 2b fiber (37). Interestingly, voluntary wheel-running was able to markedly increase the percentage of type 2a fibers while lowering the percentage of type 2b fibers without significantly changing phospholipid fatty acid profile. Data such as these provide a corresponding link between muscle fatty acid composition and fiber type, which may be important in insulin action. These data support earlier suggestions (38,39), however, that factors such as diet and exercise might work synergistically via variables such as membrane lipids and fiber type to influence insulin action.

Skeletal muscle plays a major role in insulin-stimulated glucose disposal. There is now a range of evidence in humans and experimental animals demonstrating strong relationships between the fatty acid composition of structural membrane lipids and insulin action. *In vivo* work is correlative, but *in vitro* studies suggest that a causal relationship exists. There are a number of ways that a more saturated membrane lipid profile might be obtained. It may reflect dietary fatty acid profile; it may relate to a genetic alteration in the binding and subsequent incorporation of certain fatty acids (e.g., a defect in certain FABP); it may reflect a genetic defect in one or more of the enzymes of fatty acid elongation and desaturation that are necessary to turn the major dietary fatty acids into the longer-chain, more highly unsaturated fatty acids in membrane linked to good insulin action. Equally, there are a number of ways in which a more saturated membrane lipid profile might impair insulin action: these include altered insulin receptor binding and affinity as suggested by earlier work; altered ability to translocate and insert glucose transporters, and altered intrinsic activity of those glucose transporters. Hydrolysis of phospholipids in membranes will yield diacylglycerols with differing fatty acid groups, and these might interact, in their second messenger role, quite specifically with a particular protein kinase C variant for example. Work is currently under way in both *in vivo* and *in vitro* model systems to attempt to extend the evidence for the causality of the relationship between membrane lipid profile and insulin action and also to understand mechanisms involved.

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Role of Lipids in Development of Noninsulin-Dependent Diabetes Mellitus: Lessons Learned from Pima Indians

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ABSTRACT: We studied the role of lipids in the pathogenesis of noninsulin-dependent diabetes mellitus (NIDDM) in Pima Indians. High plasma levels of nonesterified fatty acid (NEFA) predicted development of NIDDM, but this effect cannot entirely be explained by the glucose–fatty acid cycle. Dyslipidemia, although often associated with diabetes, did not seem to predict NIDDM and might rather be associated with, or the consequence of insulin resistance. In some individuals, a single amino acid substitution in the intestinal fatty acid binding protein could result in increased rates of intestinal absorption of dietary NEFA and thereby contribute to increased lipid-oxidation rates and insulin resistance.

Lipids 31, S-267–S-270 (1996).

Prospective studies conducted over the past 13 years in Pima Indians have shown that obesity (especially central adiposity), insulin resistance, and impaired insulin secretion are major risk factors for the development of noninsulin-dependent diabetes mellitus (NIDDM) (1). More than 30 years ago, Randle *et al.* (2) documented that elevated nonesterified fatty acid (NEFA) concentrations result in increased fat oxidation and decreased glucose uptake and utilization in muscle. An increased production rate of NEFA from adipose tissue could therefore be responsible for insulin resistance. Insulin resistance and NIDDM are often associated with an altered lipoprotein profile, i.e., increased very low density lipoproteins (VLDL), decreased high density lipoproteins (HDL), and altered composition of low density lipoproteins (LDL) (3–6). Surprisingly, despite the abundance of information on the competition between lipid and carbohydrate metabolism at the cellular level (7–12), no prospective study has been designed to investigate the cause-and-effect relationship between high plasma NEFA concentrations and/or dyslipidemia and NIDDM. Ongoing longitudinal studies of the development of NIDDM among Pima Indians allowed us to test whether large fat cells and high rates of *in vitro* fat-cell lipolysis are associated with high plasma fasting NEFA concentrations and whether these three factors are predictive of NIDDM. We also investigated the interaction between lipoproteins and insulin resistance in development of NIDDM. Finally, we present genetic evidence that a recently identified polymorphism in a lipid binding protein gene contributes to altered lipid metabolism and predicts insulin resistance.

Predictive role of NEFA on development of NIDDM (13). We analyzed data from annual examinations of 190 nondiabetic Pima Indians. After a mean follow-up of 4.0 ± 2.4 years (mean ± SD), 47 subjects developed NIDDM. Risk factors for NIDDM were estimated by proportional hazard analysis and risk ratios (RR) with 95% confidence intervals (95% CI) calculated at the 90th and 10th percentiles of predictor variables (Table 1). Large fat cells were predictive of NIDDM (RR = 2.4; 95% CI = 12–24.8) independent of age, sex, percent body fat, and body-fat distribution. A high fasting plasma NEFA concentration was also a risk factor for NIDDM (RR = 2.3; 95% CI = 1.1–4.7) independent of sex, percent body fat, body-fat distribution, and insulin-mediated glucose uptake (Fig. 1) but not independent of insulin secretion. Basal lipid oxidation did not predict NIDDM. The relationship between fat-cell size and NIDDM is not surprising, since the volume of adipocytes is strongly related to obesity, a major risk factor for development of NIDDM (1). It is likely that larger fat cells have higher rates of lipolysis, leading to increased plasma NEFA concentrations. This hypothesis is supported

TABLE 1
Nonesterified Fatty Acids and Development of Noninsulin-Dependent Diabetes Mellitus[a]

Factors from which NEFA effect is independent	Risk ratio	95% Confidence interval
Age, sex, percent body fat, waist/thigh[b]	2.2	1.1–4.2
Sex, percent body fat, M[c]	2.1	1.1–4.2
Sex, percent body fat, M, fat cell size	2.0	0.9–4.1
Sex, percent body fat, M, acute insulin response	2.0	0.7–5.3

[a]Risk ratios and 95% confidence intervals are presented when comparing the 90th (485 μM) and 10th (248 μM) percentiles of plasma nonesterified fatty acid (NEFA) concentrations.
[b]Waist/thigh is an index of body-fat distribution.
[c]M = Insulin-mediated glucose uptake.

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Abbreviations: *FABP2*, intestinal fatty acid binding protein locus; HDL, high density lipoproteins; IFABP, intestinal fatty acid binding protein; LDL, low density lipoproteins; NEFA, nonesterified fatty acid; NIDDM, noninsulin-dependent diabetes mellitus; RR, risk ratio; SD, standard deviation; VLDL, very low density lipoproteins.

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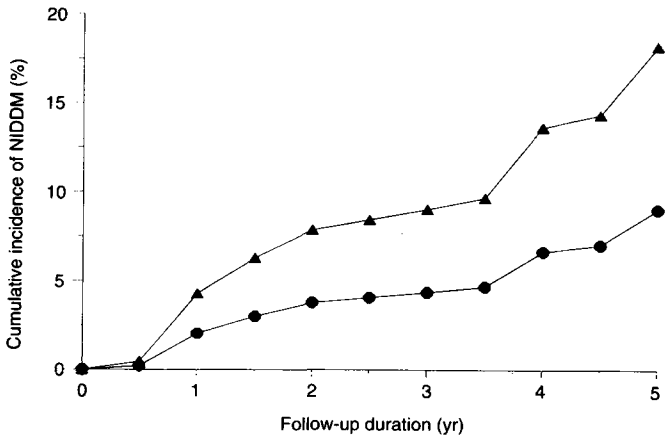


FIG. 1. Cumulative incidence (in percent) of developing noninsulin-dependent diabetes mellitus (NIDDM) for subjects at the 90th (485 μM) (▲) and 10th (248 μM) (●) percentiles of nonesterified fatty acid (NEFA) distribution. The predictive effect of NEFA is adjusted for differences in sex, percent body fat, and insulin-mediated glucose uptake (adapted from Ref. 13).

by our previous data showing that the size of the fat depot, rather than the basal rate of lipolysis *per se*, determines NEFA availability (14). In the present study, such a mechanism was also suggested by the positive correlation among average fat-cell size, basal lipolysis, and fasting plasma NEFA concentration.

The inhibitory role of NEFA on glucose disposal was proposed by Randle *et al.* more than 30 years ago (2). Subsequent clinical studies have detailed the physiological role of NEFA in regulating glucose as well as glycogen metabolism in peripheral tissues (12,15,16). The glucose–fatty acid cycle, however, cannot entirely explain the effect of NEFA on development of NIDDM since, in our study, (i) fasting plasma NEFA concentrations predicted NIDDM independently of insulin-mediated glucose uptake; (ii) basal lipid oxidation was not a significant predictor of NIDDM; and (iii) the predictive value of fasting NEFA concentrations on the development of NIDDM disappeared after adjusting for acute insulin secretion (Table 1). The last finding suggests that NEFA may increase the risk of NIDDM by inhibiting insulin secretion. This hypothesis is now supported by *in vitro* as well as *in vivo* data (17–19).

Circulating NEFA levels can affect liver metabolism. NEFA inhibit insulin receptor binding and tyrosine kinase activity in isolated hepatocytes (20) and provide energy for gluconeogenesis, thus increasing hepatic glucose output (21,22). Recently, Rebrin *et al.* (23) have suggested that insulin suppresses hepatic glucose output not directly, as generally believed, but via its regulatory action on plasma NEFA concentrations.

Predictive role of lipoproteins on development of NIDDM (24). The cross-sectional association between plasma lipoprotein abnormalities (high plasma VLDL, small dense LDL particles, and low HDL concentrations) and insulin resistance/diabetes has been well documented (3–6). Cross-sectional studies, however, cannot dissociate between cause and effect. The RR associated with each lipoprotein for the development of NIDDM is reported in Table 2. After adjustment for insulin-mediated glucose uptake, high LDL and low HDL plasma concentrations were no longer predictive of development of NIDDM. None of the lipoproteins were predictive of a worsening in insulin-mediated glucose uptake. Along with several other lines of evidence (25–28), the present study provides longitudinal data to support the idea that dyslipidemia is not causative of diabetes but, on the contrary, insulin resistance might be a possible determinant of the changes in lipoprotein profile in NIDDM.

Intestinal fatty acid binding protein and lipid metabolism. We have previously reported genetic linkage between measures of insulin action in Pima Indians and a region on chromosome 4q, which includes the intestinal fatty acid binding protein locus (*FABP2*) (29). The *FABP2* locus encodes the human intestinal fatty acid binding protein (IFABP), which binds dietary long-chain fatty acids and is expressed solely in the enterocytes of the proximal small intestine. We identified a polymorphism at codon 54 of this gene that results in an alanine-encoding allele (frequency 0.71) and a threonine-encoding allele (frequency 0.29). We have shown that the threonine-containing protein (Thr54-IFABP) has a twofold greater affinity for long-chain fatty acids than the alanine-containing protein (Ala54-IFABP) (30). In addition, cultured intestine-like cells expressing Thr54-IFABP transport long-chain fatty

TABLE 2
Unadjusted Risk Ratios and 95% Confidence Intervals for Fasting Plasma Lipoproteins and Development of Noninsulin-Dependent Diabetes Mellitus

	10th Percentile (mg/dL)	90th Percentile (mg/dL)	Risk ratio	95% Confidence interval
VLDL cholesterol[a]	7.0	23.0	1.2	0.7–2.1
LDL cholesterol[b]	79.0	141.0	0.7	0.3–1.6
HDL[c] cholesterol[d]	28.0	48.0	2.6	1.1–5.9
VLDL triglycerides	28.4	122.5	1.0	0.5–2.1
LDL triglycerides	22.9	64.7	3.3	1.6–7.0

[a]VLDL = Very low density lipoproteins.
[b]LDL = Low density lipoproteins.
[c]HDL = High density lipoproteins.
[d]For HDL, the 10th vs. the 90th percentile of fasting plasma concentrations were compared; for all others, the 90th vs. the 10th percentile were compared.

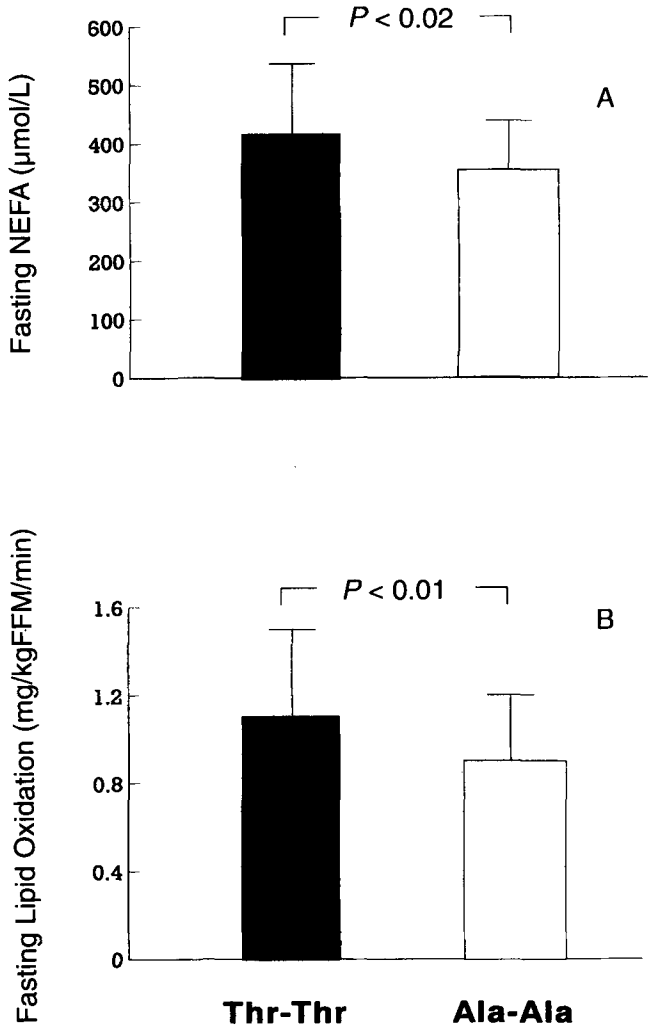


FIG. 2. A) Fasting plasma nonesterified fatty acid (NEFA) concentrations and B) lipid oxidation rates in 89 Pimas homozygous for the Thr54-intestinal fatty acid binding protein (IFABP) allele (Thr-Thr) compared to 21 Pimas homozygous for the Ala54-IFABP allele (Ala-Ala).

acid from the apical to basolateral membranes more rapidly than do cells expressing Ala54-IFABP (31). Consistent with these findings, Pimas homozygous for Thr54-IFABP had significantly higher plasma fasting NEFA concentrations and fasting lipid oxidation rates, as compared to Pimas homozygous for Ala54-IFABP (Fig. 2). Furthermore, Pimas homozygous or heterozygous for the threonine-encoding allele showed higher mean fasting insulin concentrations, higher insulin responses to oral glucose and a mixed meal, and lower insulin-stimulated glucose uptake when compared to subjects homozygous for the alanine-encoding allele (30). We are currently directly testing the hypothesis that individuals expressing Thr54-IFABP have an increased rate of transport of NEFA across the intestine compared to individuals expressing Ala54-IFABP. This may explain the association of Thr54-IFABP genotype with increased lipid oxidation rates and insulin resistance.

The role of lipids in the pathogenesis of NIDDM has been studied for more than 30 years, ever since Randle's first report of a glucose–fatty acids cycle, i.e., a competition among substrates for the oxidative pathways at the cellular level. Longitudinal studies conducted over the past 13 years in Pima Indians have shown that high plasma NEFA levels predict development of NIDDM. The effect of NEFA on NIDDM, however, cannot entirely be explained by the Randle cycle, since this effect is still present after adjusting for insulin-mediated glucose uptake. We have also shown that dyslipidemia, often associated with diabetes, does not seem to have a causative role in the development of the disease. In contrast, our analysis indicates that abnormalities in the lipoprotein profile are either associated with or a consequence of insulin resistance. Finally, we have provided evidence that a single amino acid substitution in the IFABP could result in increased rates of intestinal absorption of dietary NEFA and thereby contribute to increased lipid oxidation rates and insulin resistance.

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Arachidonic Acid Maldistribution in Obesity

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ABSTRACT: Arachidonic acid is an important regulator of cellular function via its effects on the physical properties of membranes, in its free form, or as a substrate for eicosanoids. Dietary studies indicate that its production is regulated, but the mechanisms of this regulation and factors influencing arachidonate distribution from the site of production remain to be determined. In particular, whether there is a nonoxidative fate for arachidonate once it has been released from phospholipid has yet to be determined. Variations in the arachidonate content of serum, liver, and muscle lipid fractions have been correlated with alterations in lipogenesis and insulin action, implying a role for arachidonate in fuel partitioning. Evidence for this mechanism acting systemically has been found in genetic models of obesity in rodents and also in humans. This review proposes that variation in the distribution of arachidonate between phospholipid and cholesteryl ester fractions participates in the abnormal fuel partitioning associated with some forms of genetic obesity.

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Both genetic and environmental factors play roles in the etiology of human obesity. To the extent that it is a factor in an individual, genetic variability only predisposes a person to weight gain, rather than determining the disease with certainty. Overfeeding and underfeeding studies of identical twins indicate that genetic variability is determined, in part, by varying propensities for lipid storage as opposed to its oxidation (1,2), but the biochemical mechanisms for this variability in fuel partitioning remain unknown. This paper presents the case for interindividual variability in the distribution of arachidonate (20:4n-6) between phospholipids (PL) and cholesteryl esters (CE) as an indicator of, if not an etiologic factor for, interindividual variability in fuel disposition and obesity.

In initial studies to determine if essential fatty acid (EFA) deficiency could result from extreme restrictions in fat intake during hypocaloric dieting, it was noted that obese humans had lower levels of 20:4n-6 in serum PL compared to nonobese normals (3,4). With initiation of dieting, the arachidonate in PL of obese patients quickly rose to normal and remained increased during rapid weight loss, but it rapidly declined when weight loss stopped. Thus the paucity of PL 20:4n-6 initially observed during obesity did not resolve following major weight loss (4). That this reduction in the proportion of 20:4n-6 in PL might be physiologically linked to obesity is suggested by our prior observation that a reduction in hepatocyte PL arachidonate is associated with accelerated lipogenesis and triglyceride (TG) secretion (5). Based on the observation that increased provision of fat does not increase its oxidation (6), we hypothesized that increased disposal of carbohydrate to fat would favor adipocyte lipid storage (4). Subsequently, Borkman *et al.* (7) reported that reduced 20:4n-6 in human muscle PL is associated with increased insulin resistance. Assuming that reduction in PL arachidonate in obesity is systemic (i.e., reduced muscle PL 20:4n-6 accompanies its paucity in liver), this would serve to reduce peripheral glucose disposal and concurrently enhance hepatic glucose use for lipogenesis as a secondary disposal pathway.

Using precursor/product ratios in muscle PL as an indirect measure of enzyme activity, Borkman *et al.* proposed that reduced muscle PL 20:4n-6 was due to impaired action of the Δ5-desaturase enzyme (7). Specifically, they observed that an increased proportion of 20:3n-6 and reduced 20:4n-6 was associated with insulin resistance, and from this they inferred a causal relationship between reduced action of the intervening enzyme (Δ5-desaturase) and insulin action (Fig. 1). Because arachidonic acid is predominantly produced in the liver, however, the level of its precursor in muscle is probably not the best indicator of substrate availability. Thus liver lipid fractions would be more desirable in determining precursor/product ratios, so an animal model of obesity and insulin resistance is desirable.

Two recent reports noted reduced liver PL 20:4n-6 in the obese Zucker rat (8,9), making this a good animal model to assess the relationships among fatty acids, lipogenesis, and obesity. Both reports (8,9) hypothesized impaired production of 20:4n-6 due to reduced desaturase activity, again based on precursor/product relationships in liver PL. To test the hypothesis that Δ6-desaturase is the etiologic factor causing reduced PL 20:4n-6 in this animal model, we fed lean (FaFa) and obese (fafa) weanling male Zucker rats 70 μL γ-linolenic acid (18:3n-6) by gastric gavage and compared weight gain, food intake, and percent carcass fat to analogous groups given an equal volume of soy oil by gavage (10). Following 60 days of *ad libitum* food intake, the soy oil gavage had no effect on food intake or body composition of either the lean or obese animal groups compared to chow-fed, nongavage controls. Likewise, food intake, weight gain, and per-

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Abbreviations: CE, cholesteryl esters; EFA, essential fatty acid; PL, phospholipids; TG, triglyceride.

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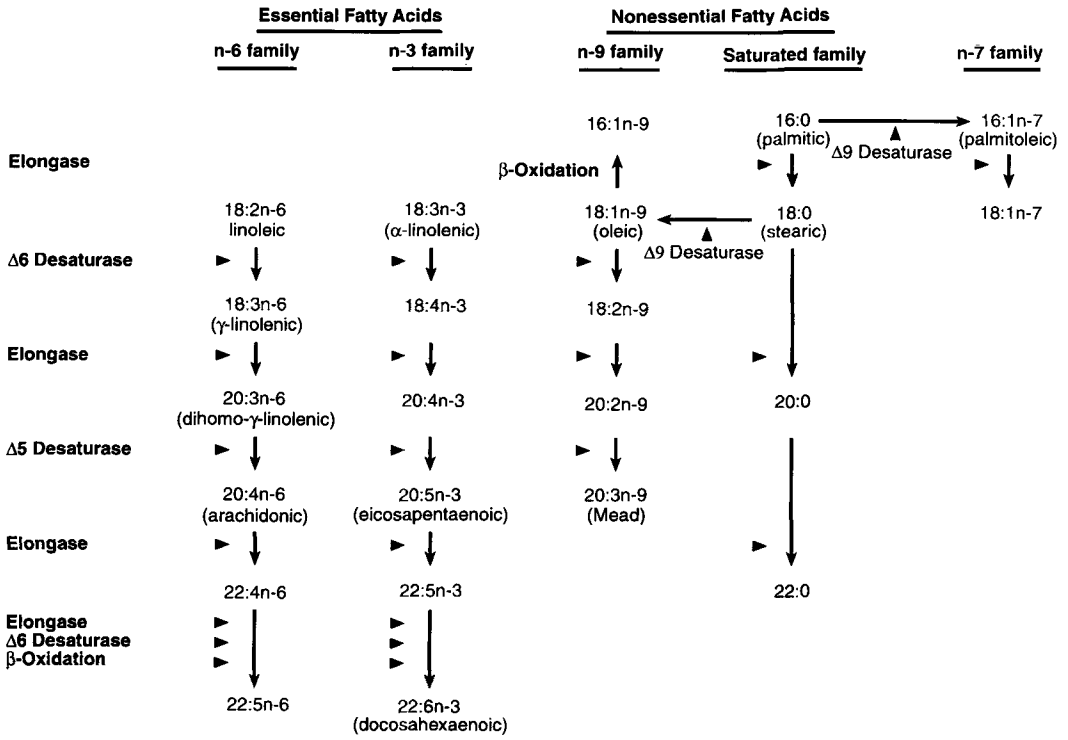


FIG. 1. Pathways involved in the endogenous modification (anabolism) of dietary fatty acids. Of particular note is the common use of the Δ6- and Δ5-desaturases in the production of the essential and nonessential highly unsaturated products 20:4n-6, 20:5n-3, and 20:3n-9.

cent carcass fat of lean animals gavaged with 18:3n-6 were no different than soy-gavaged controls. The group of obese animals gavaged with 18:3n-6, however, had markedly reduced food intake and weight gain and marginally reduced percent body fat compared to soy-gavaged obese controls. Liver PL 20:4n-6 in obese animals gavaged with 18:3n-6 was increased to normal levels by this procedure, implying that impaired Δ6-desaturase in this genotype had been overcome by the 18:3n-6 gavage. Following analysis of additional lipid fractions, however, it was noted that liver CE 20:4n-6 was dramatically increased in the soy-gavaged obese animals as compared to leans, and this excess proportion of 20:4n-6 was also seen in serum CE of both obese animal groups. An additional observation was that of marked elevation in proportions of 20:5n-3 and Mead acid (20:3n-9) in liver and serum PL and CE fractions in obese controls, with the proportions of both being reduced with 18:3n-6 administration.

Our observation of increased 20:4n-6 in liver CE of obese animals makes it unlikely that there is impaired desaturase activity (either Δ6 or Δ5) in the obese Zucker rat. While we did observe elevated proportions of both 18:3n-6 and 20:3n-6 in liver and serum PL and CE fractions in obese animals as compared to leans, an alternate explanation for their increased levels is that 20:4n-6 is being removed from PL at an accelerated rate, accumulating in the CE fraction. If PL 20:4n-6 is the feedback pool that regulates substrate entry into the fatty acid anabolic pathway (Δ6-desaturase, elongase, Δ5-desaturase), then pathway flux would be accelerated to compensate with increased 20:4n-6 production, leading to increases in its two metabolic intermediates. This would also result in enhanced production of 20:5n-3 from 18:3n-3, and 20:3n-9 from 18:1n-9. With the provision of 18:3n-6, however, the Δ6-desaturase step would be bypassed, more 20:4n-6 would be produced, and secondary product output by the anabolic pathway would be reduced. These conditions, all observed in our experiment reported in (10), are summarized here in Table 1.

Taken in total, these observations strongly support the concept of a metabolic shift (i.e., a maldistribution) of 20:4n-6 from PL to CE in the obese Zucker rat. Key to this understanding are proportionate elevations in serum and liver CE 20:4n-6 in the obese genotype, as well as increases in secondary products of the fatty acid anabolic pathway in liver and serum PL and CE fractions. That 18:3n-6 given to the obese animal nonetheless reduced food intake, weight gain, and percent body fat, while the identical dose had no effect on the lean genotype, strongly indicates an etiologic role for altered 20:4n-6 homeostasis in this animal model of genetic obesity.

The Zucker rat is a popular animal model used in the study of genetic obesity, in part because its single gene defect results in dramatic phenotypic characteristics between lean and

TABLE 1
Summary of 18:3n-6 Feeding Experiment in Obese Zucker Rats (10)

Condition	Expected result
Increased[a] 20:4n-6 loss from PL[b] to CE[c]	Reduced[a] PL 20:4n-6 Increased CE 20:4n-6 Increased fatty acid anabolic activity (i.e., increased 18:3n-6, 20:3n-6, 20:3n-9, 20:5n-3)
Increased lipogenesis due to reduced PL 20:4n-6	Increased lipogenic products (i.e., 16:0, 16:1) Decreased essential precursors (18:2n-6, 18:3n-3)
Feeding 18:3n-6 to obese genotype	Increased liver 20:4n-6 in PL Reduced secondary products (20:3n-9, 20:5n-3) Reduced lipogenesis and body fat

[a]Increased and reduced are relative to lean control animals fed soy oil.
[b]PL = phospholipids.
[c]CE = cholesterol esters.

obese animals. It differs markedly from the human disease, however, in that the latter is thought to result from variations in multiple genes. To assess potential for a distributional abnormality in 20:4n-6 in a multigenic animal model of obesity, we determined liver lipid fraction fatty acid composition from BSB mice ranging in percent body fat from 1 to 48% (11). In this animal model of obesity, unlike our finding in the Zucker rat, there was no correlation between liver PL 20:4n-6 and percent carcass fat. There was a strong positive relationship, however, between CE 20:4n-6 and carcass fat, consistent with our hypothesis of diversion of arachidonate out of the PL pool. In addition, liver PL Mead acid and 20:5n-3 were also increased in the animals with the greater percent carcass fat, supporting the concept that there is increased production of 20:4n-6 in obesity via an activated fatty acid anabolic pathway, with parallel increases in secondary products such as 20:5n-3 and Mead acid. Thus 20:4n-6 also appears to be maldistributed into the CE fraction in this animal model, but the varying degrees of reduced 20:4n-6 in PL between BSB mice and Zucker rats suggest genotype or species differences. The physiological importance of these observations in the BSB mouse is reinforced by the observation that liver CE 20:4n-6 is correlated with fasting plasma insulin (12), implying that the shift of 20:4n-6 from PL into CE is involved with impaired insulin action in the periphery.

Although increased proportions of Mead acid and reduced linoleic acid are seen in multiple lipid fractions of obese rats and mice (10,11), this should not be taken as a sign of EFA deficiency. Mead acid appears to be increased due to overall increased fatty acid anabolic pathway flux [also seen in human vegetarians (3)]. Reduced 18:2n-6 is secondary to the dilutional effects of lipogenic products such as 16:0, 16:1, and 18:0 entering the hepatic TG pool.

Another consistent finding across species is elevated 20:3n-6 with obesity. This is consistent with the observations of others (13) and viewed in isolation could be interpreted as due to reduced Δ5-desaturase activity in obesity. Pending cloning of the Δ5-desaturase gene such that we can directly quantitate its expression, this issue cannot be definitely resolved. The increased proportion of Mead acid and 20:5n-3, however, as well as the consistent observation of excess 20:4n-6 in CE fractions, strongly supports the alternative view that maldistribution rather than impaired production is extant. In this context, reliance on precursor/product ratios derived for one fatty acid family in a single PL pool should be interpreted with caution.

Based on the described animal experiments, the most consistent changes in fatty acid distribution in obesity are accumulation of 20:4n-6 in liver CE and increases in 20:5n-3 and 20:3n-9 in all PL pools. In humans with variable intakes of 18:3n-3 and in animal models fed oil low in 18:3n-3 (such as corn, olive, or safflower), however, a secondary increase in 20:5n-3 is unlikely. In addition, it appears that serum levels of 20:4n-6 in PL and CE are very sensitive to energy balance, rising with energy restriction and possibly declining with overfeeding. This variability makes serum fatty acids a less-than-consistent biomarker of obesity when energy intake is not effectively controlled.

Another important concern with our hypothesis of altered arachidonic acid distribution in genetic obesity is that it is observed across species involving a variety of separate genetic loci. In view of the central role played by arachidonic acid in regulation of cellular and organ function, however, it is likely that many genetic loci will participate in the regulation of its production and distribution. If these factors in turn influence a variety of parameters of carbohydrate and lipid fuel partitioning (e.g., lipogenesis and insulin resistance), the result could be an elegant pattern of varied energy efficiencies within a species in which multiple genes are involved. Indeed, the alterations in fuel partitioning induced by growth hormone may be due in part to alterations in the production and distribution of arachidonate (14).

In summary, these observations suggest that dynamics of 20:4n-6 production and distribution are important in regulation of lipogenesis, insulin action, and obesity in animal models and in humans. While direct measurements of 20:4n-6 flux have not been made, increased levels of secondary products such as 20:5n-3 and Mead acid can be used to infer activity of the fatty acid anabolic pathway. This, in combination with elevated proportions of 20:4n-6 in CE, supports the concept that desaturase activities are not significantly impaired in obesity. Molecular mechanisms resulting in distributional abnormalities of arachidonic acid in genetic obesity remain to be elucidated.

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Gender Differences in Insulin-Induced Free Fatty Acid Suppression: Studies in an African American Population

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ABSTRACT: This study of African Americans (AA) was designed to investigate gender differences in insulin-induced free fatty acid (FFA) suppression. Sixty AA (34 women, mean age 34 ± 7.6 years, and 26 men, mean age 30 ± 2.9 years) participated. All subjects had an oral glucose tolerance test (OGTT). Nineteen women and 18 men also underwent a euglycemic hyperinsulinemic clamp (IC) study. Plasma insulin and FFA concentrations were obtained during both tests at 0, 60, and 120 min. While there was no gender difference in body mass index ($P = 0.21$), women had greater percent body fat ($P < 0.001$) calculated by the Siri formula. There was no gender difference in fasting FFA levels, but during the OGTT, women compared to men had significantly greater FFA suppression. Both nonobese and obese women suppressed FFA concentration by 88%, and nonobese and obese men suppressed FFA concentration by 80 and 66%, respectively. This gender difference in FFA suppression was significant ($P = 0.001$) and independent of obesity and insulin concentration. During the IC studies, there were no gender or obesity differences in FFA suppression, with women and men suppressing FFA levels by 87–89% ($P = 0.7$). Fasting insulin concentrations were higher in obese vs. nonobese ($P = 0.03$), but fasting FFA concentrations were not different ($P = 0.15$). For nonobese and obese females, fasting FFA levels were 0.55 ± 0.24 and 0.44 ± 0.26 mEq/L, respectively, and for nonobese and obese males, 0.45 ± 0.2 and 0.35 ± 0.18 mEq/L, respectively. In women, development of obesity may be enhanced by greater sensitivity to insulin-induced FFA suppression as measured during an OGTT. To detect gender differences in FFA metabolism, the OGTT is superior to the IC. The lack of elevation in fasting FFA levels in obese AA women and men has not been reported in other racial groups and may indicate a greater adipocyte sensitivity to insulin in AA.

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African American (AA) women have a higher prevalence of obesity than do AA men (1). After the age of 25, 34% of AA women are obese, compared to 18% of AA men, and after the age of 45, 61% of AA women are obese, compared to 41% of AA men (1). Obesity in AA women is independent of socioeconomic status (1).

To better understand the metabolic basis for the development and maintenance of this excess obesity in AA women, adipocyte sensitivity to insulin could be studied (2). As free fatty acid (FFA) release by adipose tissue is a major determinant of FFA levels (3,4), *in vivo* adipocyte sensitivity to insulin can be assessed by obtaining FFA concentrations during an oral glucose tolerance test (OGTT) (5) or a hyperinsulinemic clamp (IC) (3,4). It is unknown whether an OGTT or an IC has greater sensitivity in studying gender differences in FFA metabolism.

This study was designed to determine if there are gender differences in insulin-induced FFA suppression in AA.

MATERIALS AND METHODS

Sixty clinically well, nondiabetic AA (34 premenopausal women, mean age 34 ± 7.6 and 26 men, mean age 30 ± 2.9, age range 21–37) participated. Obesity was defined as a body mass index (BMI) ≥ 28 (kg/m^2). Anthropometric measures were performed (arm circumference, skinfold thicknesses). From anthropometric measures, using the Siri equation, percent body fat and total body fat were calculated (6). Each subject had a 75 g OGTT. Thirty-seven of the participants (19 women and 18 men) also underwent a 2-h euglycemic IC, performed according to previously described methods (7). During both the OGTT and IC, plasma FFA and insulin concentrations were determined at 0, 60, and 120 min. Plasma FFA levels were determined by the enzymatic colorimetric method (Wako NEFA C test, Osaka). Plasma insulin concentrations were determined with a solid-phase radioimmunoassay (Coat-A-Count, Diagnostic Products Corporation, Los Angeles). The protocol of the study was approved by the Institutional Review Board of the Medical College of Pennsylvania. Statistical differences between obesity groups and gender were determined using a two-way analysis of variance (ANOVA) with a test for interactive effects. All values are expressed as mean ± SD unless otherwise indicated.

RESULTS

The mean age of the women was 4 years higher than that of the men, but all the women were premenopausal and menstruated regularly. BMI was similar in women and men (30.2 ±

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Abbreviations: AA, African American; ANOVA, analysis of variance; BMI, body mass index; FFA, free fatty acids; IC, hyperinsulinemic clamp; OGTT, oral glucose tolerance test.

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TABLE 1
Anthropometrics[a]

	Nonobese women (n = 15)	Men (n = 15)	Obese women (n = 19)	Men (n = 11)	Two-way ANOVA[b] Gender (F,P-Values)	Obesity (F,P-Values)	Interaction (F,P-Values)
Weight (kg)	67 ± 9	77 ± 13	96 ± 12	98 ± 11	3.4 0.07	63, <0.001	1.3, 0.26
BMI (kg/m^2)	24 ± 3.4	24 ± 2.7	35 ± 4	33 ± 4	1.6, 0.21	122, <0.001	1.9, 0.16
Arm circ. (cm)[c]	29 ± 2.2	31 ± 3.6	38 ± 5.1	37 ± 3.6	0.38, 0.54	43, <0.001	0.91, 0.35
TSF (mm)[d]	21 ± 5.7	12 ± 6	27 ± 6	19 ± 5.1	30.0, <0.001	15, <0.001	0.2, 0.66
SSSF (mm)[e]	22 ± 7	16 ± 8	36 ± 10	29 ± 8	7.5, 0.009	34, <0.001	14.0, < 0.001
%Body fat	34 ± 4	18 ± 7	39 ± 4	27 ± 3	127.0, <0.001	34, <0.001	54.0, < 0.001
Total body fat (kg)	23 ± 4	15 ± 7	38 ± 7	27 ± 5	31.0, <0.001	64, <0.001	0.65, 0.42

[a]Values are the mean ± SD. BMI, body mass index.
[b]Two-way analysis of variance (ANOVA) was used to determine statistical differences between gender and obesity groups with a test for interactive effects.
[c]Arm circumference.
[d]Triceps skinfold.
[e]Subscapular skinfold.

6.9 vs. 27.6 ± 5.6 kg/m^2, respectively, $P = 0.12$) but women had significantly greater body fat in kilograms (30.4 ± 10.2 vs. 20.8 ± 8.5, $P = 0.001$) and percent body fat (36.1 ± 5.9 vs. 22.8 ± 6.6, $P = 0.001$). In Table 1 anthropometrics are provided by gender within each obesity group, that is, BMI ≤ 28 or ≥ 28. By BMI and arm circumference, women and men were the same size, but by skinfold thicknesses, % body fat, and total body fat in kilograms, women were significantly more obese.

Women and men had similar fasting insulin and FFA concentrations (Fig. 1). During the OGTT, at 120 min women had greater percent suppression of FFA ($P = 0.001$) (Fig. 2A). This gender difference was independent of obesity and insulin concentrations. During the IC, there was no gender difference in percent suppression of FFA (Fig. 2B). The area under the insulin curve was similar during the OGTT and IC. This means that gender differences seen during the OGTT vs. the IC cannot be attributed to differences in insulin concentrations between these two types of tests.

When obese subjects were compared to the nonobese, the obese had higher fasting insulin concentrations ($P = 0.03$) (Fig. 1A). Obese subjects were also noted to have lower fasting FFA concentrations than the nonobese, with this difference approaching significance ($P = 0.08$) (Fig. 1B).

DISCUSSION

Examination of the effect of insulin on serum FFA levels may help determine the metabolic basis for the high prevalence of female obesity in AA. Insulin affects FFA concentration in three ways: first, by inhibiting lipolysis; second, by promoting reesterification, and third, by decreasing FFA oxidation. Of these three factors, the major effect of insulin appears to be to regulate a balance between lipolysis and reesterification (3,4,8).

We found that, despite similar BMI in women and men, women had significantly greater body fat content (Table 1). During the OGTT, women suppressed FFA concentration significantly more than did men ($P = 0.001$). This gender difference was independent of obesity and insulin concentration. Therefore, if women and men have similar insulin concentrations but women suppress more, then women appear more

FIG. 1. A) Fasting insulin concentrations during the oral glucose tolerance test (OGTT). The number of women and men in each group is provided in Table 1. Data are presented as means ± SE, with data analyzed by two-way analysis of variance. Fasting insulin concentration was significant by obesity group ($P = 0.03$) but not by gender ($P = 0.13$). B) Fasting free fatty acids (FFA) concentrations during the OGTT. Data are presented and analyzed as described for A). Fasting FFA concentration by obesity group approached significance at $P = 0.08$. Gender difference was not significant ($P = 0.15$).

FIG. 2. A) Percent suppression of FFA during the OGTT. The number of women and men in each group is provided in Table 1. Data are presented as means ± SE. Data was analyzed by two-way analysis of variance (ANOVA). Gender difference in percent suppression of FFA was significant ($P = 0.001$). B) Percent suppression of FFA during the hyperinsulemic clamp (IC). Data are presented and analyzed as described for Figure 1A. The number of subjects in each group for the IC is as follows: nonobese women (10) and men (8) and obese women (9) and men (10). By two-way ANOVA, gender difference in percent suppression of FFA was not significant ($P = 0.7$). Abbreviations as in Figure 1.

sensitive to insulin-induced FFA suppression than men. Because insulin is a profoundly lipogenic hormone, this increased sensitivity to insulin in women could promote development and maintenance of obesity.

In contrast to the gender difference seen in FFA suppression during the OGTT, there was no gender difference with the IC ($P = 0.7$) (Fig. 2B). As men achieve maximum suppression during the IC, men do not appear to have a defect in insulin responsiveness (9). For men during the IC, exposure to a primed continuous intravenous infusion of exogenous insulin appears to overcome resistance to insulin secreted endogenously during the OGTT. Therefore, to study gender differences in FFA metabolism, the OGTT may be superior to the IC.

AA women are more obese than are AA men (1). There is a suggestion in our findings, however, that both AA men and women are more sensitive to insulin-induced FFA suppression than are other racial groups. If true, this insulin sensitivity to FFA suppression could promote obesity in AA men as well as in AA women. Racial differences in body size were found in the NHANES II (National Health and Nutrition Examination Survey II) data, which showed that, between the ages of 35 and 55, AA men are heavier than Caucasian men (1). Further, after the age of 12, female obesity is twice as common in AA as compared to Caucasians (1). In London, McKeigue *et al.* (5) performed 75 g OGTT with FFA concentrations determined at 0 and 120 min in 632 Europeans and South Asians. They found significant gender differences, but no racial differences. In their study, women suppressed FFA levels by 69%, and men by 55% ($P < 0.001$). AA women in our study suppressed FFA by 88%, and men suppressed 69–80%, depending on their obesity group (Fig. 2A). While FFA concentrations in our two studies were measured by different techniques, and the London study combined both the obese and nonobese, the degree of difference in FFA suppression in our two studies suggests there may be true population differences.

We found that obese AA had lower fasting FFA concentrations than did the nonobese (Fig. 1B). This difference approached significance at a P value of 0.08 and was present despite a significantly higher fasting insulin concentration in the obese ($P = 0.03$) (Fig. 1A). Studies that compared fasting FFA concentrations of obese and nonobese subjects reported higher FFA in the obese (2,10–15). These reports resulted from studies conducted on small samples of subjects ranging from 12 to 31. Our study population was considerably larger, composed of subjects within a narrow age range, and was clinically well.

Because we did not perform kinetic studies, we cannot determine the metabolic basis for the lower fasting FFA concentrations in obese AA subjects. In kinetic studies (4,8), it appears that differences in FFA levels between individuals are secondary predominantly to differences in rates of reesterification. Differences in rates of adipocyte antilipolysis are not as likely to be factors because antilipolysis in both the obese as well as the nonobese has been shown to be exquisitely insulin-sensitive (2,8,10). If differences in FFA regulation are based on differences in reesterification, then our obese subjects would have enhanced rates of reesterification. This is in contrast to obese subjects studied by Nestel *et al.* (8) with higher fasting FFA and decreased rates of reesterification. We know of no *in vivo* FFA kinetic studies in AA, but there is substantial evidence to suggest that FFA metabolism may be different in AA than in Caucasians. First, in autopsy studies in New Orleans where epidemiologic evidence suggests that the dietary fatty acid intake was similar in AA and Caucasians, fatty acid composition of their adipose tissue was different (16). Second, in *in vitro* studies of adipose tissue obtained by biopsy from those of Africans living in Canada and Caucasian Canadians, significant racial differences in sensitivity to epinephrine induced lipolysis were demonstrated (17). Yet *in vitro* studies are inadequate predictors of *in vivo* FFA metabolism precisely because *in vitro* studies cannot measure rates of reesterification (18). Therefore, to determine metabolic mechanisms for the lower FFA concentrations in obese AA *in vivo* turnover studies must be performed.

In conclusion, greater suppression of FFA during the OGTT in women compared to men suggests a greater adipose tissue sensitivity to insulin, which could promote development and maintenance of female obesity. To determine gender differences in insulin suppression of plasma FFA concentrations, the OGTT may be superior to the IC. As obese AA did not have elevated fasting plasma FFA concentrations, as reported in other populations (2,10–15), AA may have a greater adipocyte sensitivity to insulin.

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Trans, n-3, and n-6 Fatty Acids in Canadian Human Milk

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ABSTRACT: The presence of *trans* fatty acids in human milk may be a concern because of their possible adverse nutritional and physiological effects on the recipient infant. The mother's diet is the source of human milk *trans* fatty acids, and since these fatty acids are prevalent in many common foods of the Canadian diet, the *trans* fatty acid content and the fatty acid composition of Canadian human milk were measured by gas–liquid chromatography coupled with silver nitrate–thin layer chromatography. In samples obtained from 198 lactating mothers across Canada, the average percentage of total *trans* (sum of *t*18:1, *t*18:2, and *t*18:3) was 7.2% of breast milk fatty acids with a range of 0.1–17.2%. Analysis of *t*18:1 isomer distribution indicated that partially hydrogenated vegetable oils are the major source of these *trans* fatty acids in human milk, whereas contribution from dairy products appeared to be relatively minor. Linoleic and α-linolenic acid levels were inversely related to the total *trans* fatty acids, indicating that the elevation of *trans* fatty acids in Canadian human milk is at the expense of n-3 and n-6 essential fatty acids. Levels of arachidonic and docosahexaenoic acids did not correlate with their parent fatty acids, indicating that it might be difficult to elevate the levels of n-6 and n-3 C_{20-22} polyunsaturated fatty acids in breast milk by increasing levels of linoleic and α-linolenic acids in the mother's diet.
Lipids 31, S-279–S-282 (1996).

It is well established that *trans* and other unusual isomers of oleic and linoleic acids (1–4) that originate primarily from partially hydrogenated vegetable oil (PHVO) are transferred to human milk through maternal diets and subsequently passed on to infants (5). Several clinical studies have indicated that *trans* fatty acids (TFA) may exert some negative biological effects on the recipient infants (6–8). Particularly, TFA seem to impair the desaturation and elongation of linoleic and α-linolenic acids to their C_{20} and C_{22} polyunsaturated fatty acid (LCP) metabolites and early growth of human infants (5–8). n-6 and n-3 LCP metabolites are essential membrane components, and adequate supply of them may be required for synthesis and maturation of the central nervous system during late fetal and early postnatal growth (9).

Large amounts of PHVO are being used in Canada. For example, in 1993, Canada deodorized 807,000 tons of edible oil (mainly canola, followed by soybean), of which approximately 58% was hydrogenated for use in the manufacture of margarine (156,000 tons) and other shortenings (313,000 tons) (10). Shortenings are used in a variety of applications, including deep-fat frying in restaurants and in common bakery foods (breads, cakes, cookies, crackers, pastries, and so forth). This widespread use of PHVO in adult diets prompted us to determine the *trans* and other fatty acids in Canadian human milk. Detailed reports of this study have been published elsewhere (11,12).

MATERIALS AND METHODS

Samples of mature milk (3–4 wk after parturition) were collected from 198 lactating women across Canada. Donors were requested to express about 3–4 mL of their milk manually during each feeding starting from the very first feeding to the last feeding of the day. The collection from each donor thus represented her daily milk sample. Fat from a 5-g milk sample was extracted with $CHCl_3$-MeOH (2:1) and methylated with BF_3-MeOH. Fatty acid composition was determined by capillary (gas–liquid) GLC (SP-2560 flexible fused silica capillary column, 100 m × 0.25 mm i.d.; Supelco, Inc., Bellefonte, PA) after separating the *t*18:1 and *c*18:1 groups by $AgNO_3$-TLC (11). Positional and geometrical isomers of oleic and linoleic acids were identified as described previously (2).

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Abbreviations: $AgNO_3$-TLC, silver nitrate thin-layer chromatography; FA, fatty acids LCP, C_{20} and C_{22} n-6 and n-3 polyunsaturated fatty acids; PHCO, partially hydrogenated canola oil; PHSO, partially hydrogenated soybean oil; PHVO, partially hydrogenated vegetable oil; TFA, *trans* fatty acid.

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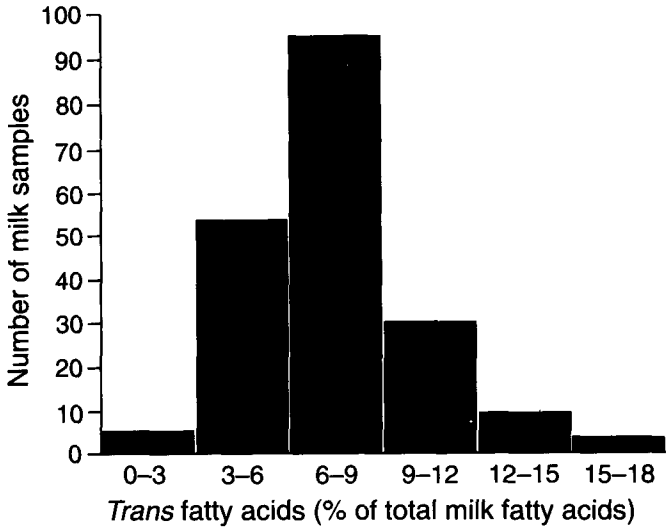


FIG. 1. Frequency distribution for *trans* fatty acid in Canadian human milk.

RESULTS

There was a wide variation (Fig. 1) in TFA content among the 198 samples, ranging from 0.1 to 17.2% of the total milk fatty acids, with a mean of 7.2% (Table 1). The most frequent occurrence (48% of the samples) was in the range of 6–9% TFA, while 22% contained more than 9% TFA. Only 6 samples contained very low levels (<3%) of TFA. As expected, the major TFA group was isomers of *t*18:1, which accounted for 5.9% of the total fatty acids. *Trans* isomers of linoleic acid were the other important group, amounting to 0.9% with 9*c*,13*t*-18:2 being the most prevalent, followed by 9*c*,12*t*-18:2, and 9*t*,12*c*-18:2.

The average *t*18:1 isomer profile of the milk samples was remarkably similar to those of partially hydrogenated canola oil (PHCO) and partially hydrogenated oil soybean (PHSO) basestocks, but it was different from that of cow milk fat (Fig. 2). Basestocks analyzed here are representative of what is available on the Canadian market.

There was no correlation among the levels of linoleic acid, α-linolenic, arachidonic, docosahexaenoic, and n-6 and n-3 long-chain polyunsaturated fatty acid (LCP) groups. Linoleic ($r = -0.29$, $P < 0.0001$) and α-linolenic ($r = -0.25$, $P < 0.001$) acids, however, were inversely correlated to *t*18:1. In addition, *t*18:1 positively correlated with stearic acid ($P < 0.01$), but no correlation was found with oleic acid.

DISCUSSION

A disturbing feature of Canadian human milk in this study is the elevated TFA level averaging 7.2% of total fatty acids, which is about 1.5- to 3.6-fold higher than values reported for many other populations (5, 13–15). These considerable differences presumably reflect a more marked prevalence of TFA in the Canadian diet. Similar high levels of TFA were also reported for Canadian human milk samples in 1976 (16), which may indicate that the TFA level in the Canadian diet has not changed over the last 18 years.

The close similarity of the *t*18:1 isomer distribution to that of PHVO shows that the major source of TFA and other isomeric fatty acids in Canadian human milk is PHVO, whereas the contribution from dairy products is minor. The 9*c*,13*t*-18:2, 9*c*,12*t*-18:2 and 9*t*,12*c*-18:2 isomers detected here are also the main linoleic acid isomers in PHVO (2), margarine (3), and bakery products (4). The very similar distribution of *t*18:1 and *t*18:2 isomers between human milk and PHVO suggests that dietary isomeric fatty acids are incorporated into human milk with no or very little alteration of their relative proportions or structures.

It is generally believed that dietary isomers of linoleic acid are not desaturated and chain-elongated to C_{20} metabolites in either humans or experimental animals consuming an adequate intake of linoleic acid (17). We have shown, however, that, in rats given PHCO with adequate levels of essential fatty acids, the above-mentioned three major *t*18:2 isomers were desaturated and elongated to their corresponding C_{20} trans polyunsaturated fatty acids (18). These metabolites, however, were not detected in human milk, perhaps because the level of linoleic acid of the mothers' diet was sufficient to prevent further metabolism of the *trans* isomers. Nevertheless, the possibility of formation of the unusual C_{20} *trans* metabolites in infants receiving human milk, particular-

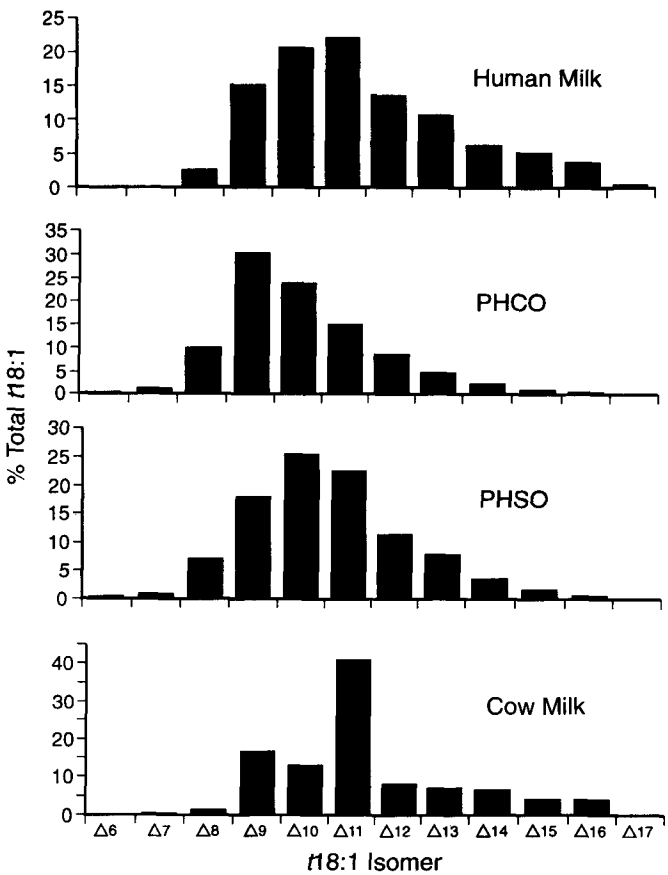


FIG. 2. Average *t*18:1 isomer distribution in human milk fat ($n = 198$), partially hydrogenated canola oil (PHCO: blend of six base stocks of iodine value varying from 64 to 92), partially hydrogenated soybean oil (PHSO; blend of six base stocks of iodine value varying from 63 to 109), and cow milk ($n = 10$).

TABLE 1
Fatty Acid (FA) Composition (wt% total fatty acids) of Pooled 24-h Collection of Mature Breast Milk from 198 Canadian Mothers

Saturated FA	Mean ± SD[a]
12:0	5.68 ± 2.01
14:0	6.10 ± 1.73
16:0	18.30 ± 2.25
18:0	6.15 ± 0.97
Total saturated FA	38.50 ± 2.94
cis-Monounsaturated FA	
16:1	2.68 ± 0.62
9*c*-18:1	30.65 ± 2.66
11*c*-18:1	1.91 ± 0.17
12*c*-18:1	0.74 ± 0.23
Total other *c*-18:1	1.57 ± 0.20
Total *cis*-monounsaturated FA	38.57 ± 4.10
Polyunsaturated FA	
18:2n-6	10.47 ± 2.62
20:4n-6	0.35 ± 0.11
Total n-6 LCP	0.83 ± 0.28
18:3n-3	1.16 ± 0.37
22:6n-3	0.14 ± 0.10
Total n-3 LCP	0.33 ± 0.24
Trans FA	
14:1	0.09 ± 0.05
16:1	0.18 ± 0.08
18:1	5.87 ± 2.52
9*t*,12*t*-18:2	0.05 ± 0.08
9*c*,13*t*-8*t*,12*c*-18:2	0.36 ± 0.14
9*c*,12*t*-18:2	0.29 ± 0.15
9*t*,12*c*-18:2	0.24 ± 0.12
18:3	0.11 ± 0.08
Total TFA[c]	7.19 ± 3.03
Unusual *cis*-polyunsaturated FA	
9*c*,15*c*-18:2	0.07 ± 0.05
8*c*,14*c*-20:2	0.19 ± 0.08
5*c*,11*c*,14*c*-20:3	0.02 ± 0.02
5*c*,8*c*,11*c*-20:3	0.05 ± 0.06
5*c*,8*c*,14*c*-20:3	<0.005
Unidentified FA	2.24 ± 0.09

[a]SD = Standard deviation.
[b]LCP = C_{20} and C_{22} n-6 and n-3 polyunsaturated FA.
[c]TFA = *trans* fatty acids.

ly those containing elevated levels of 18:2 isomers and lower levels of linoleic acid, should not be ruled out.

In addition to the isomeric fatty acids of PHVO origin, Canadian milk showed the presence of two other minor unusual C_{20} LCP 8*c*,14*c*-20:2 (0.19%) and 5*c*,8*c*,14*c*20:3 (<0.005%). These are not known to be present in dietary fats, but are most likely endogenously synthesized from dietary 12*c*-18:1 by alternative desaturation and elongation, as was observed in rats fed PHVO (18). In human milk analyzed here, 12*c*-18:1 accounted for 0.74% of the total fatty acids. The presence of the two C_{20} metabolites of 12*c*-18:1 in human milk suggests that humans are also capable of metabolizing isomeric fatty acids of PHVO origin.

The present study demonstrated an inverse correlation of TFA with linoleic and α-linolenic acids. We have also observed a similar correlation between TFA and linoleic acid in subcutaneous adipose tissue of Canadians (19). Further, in human milk there was no correlation of TFA with oleic acid, but there was a positive correlation with stearic acid. These relations reflect the prevalence of PHVO in the Canadian diet, because, during hydrogenation, TFA and to a lesser extent stearic acid are formed with concomitant reduction in linoleic and α-linolenic acids with practically very little change in the level of oleic acid (1). Thus it could be suggested that lower intakes of essential fatty acids by Canadians are invariably associated with high intakes of TFA.

Levels of linoleic (10.5%) and α-linolenic (1.2%) acids in Canadian milk were comparable to those reported in studies of mature human milk from women following *ad libitum* diets in different regions of the world, but their n-6 (20:4n-6, 22:4n-6, and 22:4n-6) and n-3 (20:5n-3, 22:5n-3 and 22:6n-3) LCP metabolites were lower (20). The lack of correlation between the two parent n-6 and n-3 essential fatty acids and their main metabolites, 20:4n-6 and 22:6n-3, was noted by others (15,21). These findings may indicate that low LCP levels in human milk in Canada cannot be restored by increasing dietary levels of linoleic and α-linolenic acids.

In summary, our findings suggest that breast milk in Canada generally has a high level of TFA and a low level of LCP. In view of the possible inhibitory effects of TFA on essential fatty acid metabolism (6–9) and the importance of LCP for the developing brain and retina (9), the combination of high levels of TFA and low levels of LCP in milk is a cause for concern, and its possible physiological consequences on infants should be investigated.

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Characteristics of Fats in Japanese Diets and Current Recommendations

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ABSTRACT: Although there is no firm evidence to support the "ideal" or even "appropriate" healthy level of dietary fat, the habitual fat consumption pattern in Japan seems to be a criterion for the recommended allowance both in the quantitative and qualitative points of view as judged from the life expectancy and the incidence of degenerative diseases. The new recommended dietary allowance of Japan, fifth revision effective for five years starting in 1995, adopted dietary fat levels of 20–25 energy percent, the ratio of saturated, monounsaturated, and polyunsaturated fatty acids at 1:1.5:1 and the ratio of n-6/n-3 at 4. The recommended fat level is similar to that previously consumed in Japan, and is even lower than that in diets used to treat hyperlipidemia in Western countries, current recommendations in those countries being 30 energy percent fat. Convincing data for the beneficial effects of n-3 polyunsaturated fatty acids on human health, in particular for healthy people, have been presented in only a few reports. However, the recommended n-6/n-3 ratio of 4 seems reasonable compared with the ratio of around 10 in other developed countries. In this context, it is more important to fully understand the nutritional and physiological roles of fat in healthy people rather than in those with chronic disease. At present, the low-fat dietary pattern in Japan appears to be a healthy way of eating.

Lipids 31, S-283–S-286 (1966).

The people of Japan are now the world's most long-lived. Life expectancy has risen at an unexpected rate in the past two or three decades. The average expected life span of babies born in 1993 is now 82.5 yr for women and 76.3 yr for men. Altered patterns of dietary intake appear to be a crucial factor in this increase. Of dietary components, changes in the dietary patterns of fats seem to be most influential, although these changes in dietary habit have changed the pattern of disease to one similar to that seen in developed Western countries. Currently, the three major causes of death in Japan are cancer, heart disease, and cerebrovascular disease (in that order), which contribute approximately 70% of the total death (1). Nevertheless, the incidence of these diseases is considerably lower as compared with that of other developed countries. It may not be ideal, but the feeding habits, including fat consumption, in Japan seem to be a healthy way of eating, at least for this population. Fat nutrition and physiology should be taken into consideration from both the qualitative and quantitative standpoints.

Quantitative standpoints. Of the changes in Japanese diet, the most significant has been a marked increase in daily fat intake from 21 g in 1955 to 58 g per capita (approximately 25 energy %) in 1992 (Fig. 1) (2). This change accompanied the decrease in carbohydrate intake from 425 to 289 g per day per capita in the same period. In these changes the relative increase in the amount of animal fats is more marked than that of vegetable oils. The increase in the intake of animal fat parallels that of animal protein. The current dietary fat level is still far lower than that in Western countries, but the proportion of vegetable oil is higher than in those countries. The current ratio of animal-to-vegetable fat in Japan is approximately 1:1. The drastic increase in fat intake in Japan has now reached a plateau. The increase in the incidence, but not total numbers, of death from so called "adult diseases" is also diminishing.

There is no definite agreement regarding the "desirable" level of dietary fat. Although 30 energy percent is recommended in most Western countries, it appears to be more makeshift than theoretically-derived. Similarly, it is not clear if the dietary level of 25 energy percent is adequate, as the pattern of disease in Japan is now becoming Westernized. Apparently, average Japanese fat consumption is thought to be acceptable, as it is just above the upper limit of recommendation. However, it is also true that there is a considerable bias in fat intake, and more than 35% of people are consuming much more fat (above 120%) and approximately 15% are consuming much less fat (below 80%) than the median value of the recommendation, 22.5%, indicating that only half of the Japanese people are achieving the recommendation. It is not clear presently which level, either 20 or 25 energy percent, is optional.

It is not easy to control the fat intake in developed countries, presumably due to its palatability. It seems, however, crucial to define the recommended daily allowance (RDA) of dietary fat much more clearly, i.e., either 20 or 25 percent of energy rather than using that as the range, as animal experiments have shown detectable physiological differences between both extremes (3). The Ministry of Health and Welfare of Japan addressed this point, and asked nutrition specialists to define a more precise recommendation. The 25 energy per-

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Abbreviations: PUFA, polyunsaturated fatty acids; RDA, recommended daily allowance.

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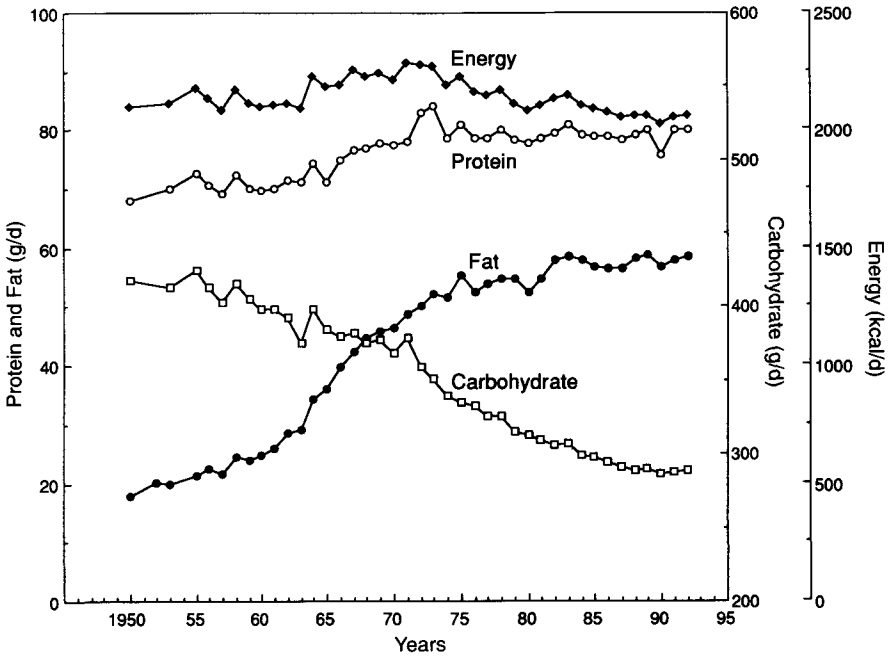


FIG. 1. Changes in nutritient intake in Japan (Ref. 2).

cent should now be regarded as the upper limit for Japan on the basis of the frequency of an increase in degenerative diseases similar to those in Western countries. The new RDA for dietary fat is summarized as follows. Quantity, 20–25 energy percent; quality: fatty acid ratio, saturated, monounsaturated and polyunsaturated fatty acids (S/M/P) = 1:1.5:1; polyunsaturated fatty acids (PUFA), n-6/n-3 = 4; and cholesterol, < 300 mg/d.

Qualitative standpoints. In the new RDA for dietary fat, the main points followed the preceding issues, but the new one simultaneously presented a preferable balance of PUFA as the n-6/n-3 ratio in addition to some revision to the balance of S/M/P.

The recommended ratio of S/M/P is 1:1.5:1. A higher value for monounsaturated fatty acids, exclusively oleic acid, was mainly based on its stability against oxidation and its abundance in regular meals in addition to avoiding the theoretical risk from increased intake of saturated fatty acids and PUFA (5).

In Japan the n-6/n-3 ratio currently recommended is 4. This value was empirically derived from the current fat intake rather than having a theoretical basis. We are consuming dietary fat at the ratio of 4:5:1 from animals, vegetables, and fish, respectively, and the n-6/n-3 ratio has apparently remained at a constant level for the past two decades (approximately 4), as shown in Figure 2 (2,6). The Japanese are consuming relatively large amounts of n-3 PUFA from fish and vegetable oils, approximately 4 g per day per capita in relation to around 15 g of n-6 PUFA. There is a decreasing trend of fish consumption in Japan, in particular in the younger generations, and the n6/n-3 ratio of their diets is apparently higher than the average value, in some cases it is above 6. However, because of the decline in ocean fishing grounds, farmed fish are now becoming more popular and available. Farmed fish in Japan have been reported to have a higher n-3 fatty acid content than do wild fish (7). Such fish are in general fatty and less expensive as well, thus unconsciously supplying a greater amount of n-3 PUFA for a lesser amount of fish.

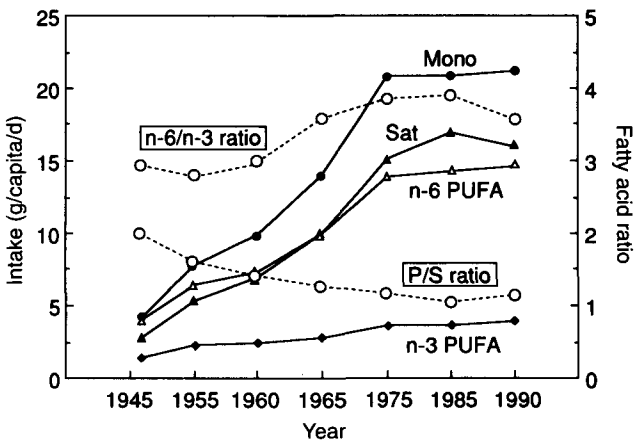


FIG. 2. Changes in the balance of fatty acid intake in Japan (Refs. 2 and 9). Ratio of linolenic acid and α-linolenic acid to their metabolites are 16:1 is 17:1, respectively. Sat, saturated; Mono, monounsaturated; PUFA, polyunsaturated fatty acids.

In addition to the high habitual fish intake in Japan, our fat source also seems to be partly responsible for a relatively high intake of n-3 PUFA. The edible fats are contributing approximately 25% of total fat intake, and two vegetable oils, rape-

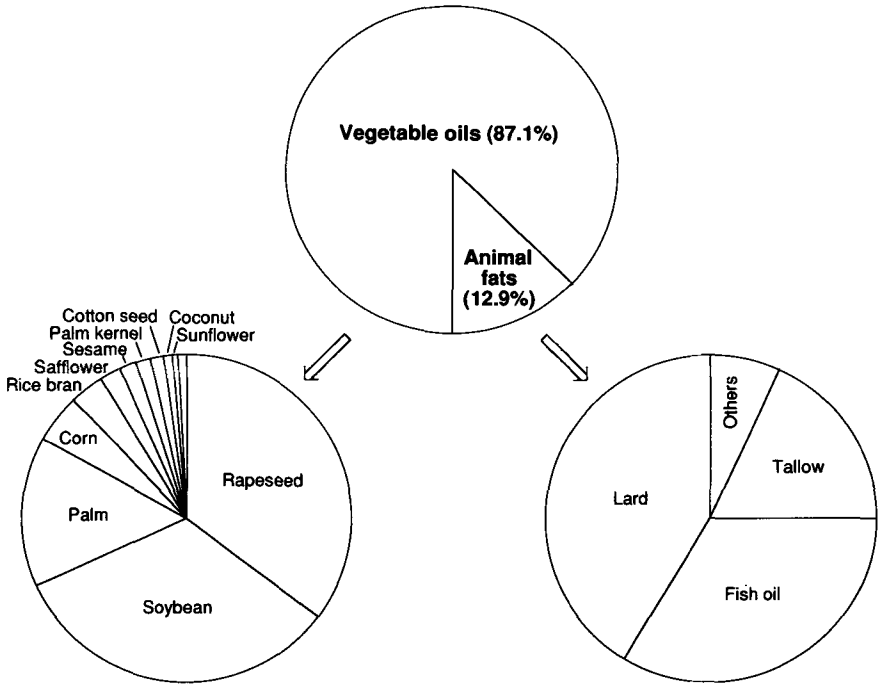


FIG. 3. Current status of the sources of dietary fats in Japan in 1993 (Ref. 8).

seed oil and soybean oil, which are exclusively α-linolenic acid-containing edible oils, account for more than 60% of total vegetable oils consumed, thus contributing a reasonable amount of n-3 PUFA (Fig. 3)(8).

The RDA considered that the n-6/n-3 ratio of 4 is acceptable for health, or at least there is no firm reason to reject this value. Some countries are now recommending a specific n-6/n-3 ratio in their RDA (9). However, at present no human data are available to indicate the optimal balance of these two series of PUFA, although a large number of animal studies are providing evidence indicating a range of beneficial physiological effects of n-3 PUFA in relation to n-6 PUFA (10,11). The proposed recommendation in most countries is between 4–10. In Western countries, where the current balance is estimated to be around 10, it would be impractical and difficult to lower the ratio to around 4.

It is also interesting that the consumption of *trans* fatty acids in Japan is very low and seldom exceeds 2 g per day per capita compared with aroung 10 g in Western countries. This was mainly attributable to the wide use of palm oil as a commercial cooking vehicle instead of the partially hydrogenated fats. In Western countries, partially hydrogenated vegetable oil is, in most cases, the exclusive source of the cooking oil in processed foods, causing a higher level of *trans* fatty acid intake. Recent human studies suggest that *trans* fat at a relatively high dietary level, in contrast to palm oil (12), worsens plasma lipoprotein profiles more than saturated fatty acids (13).

The second most important change influencing the composition of dietary fat in Japan is in the increased consumption of animal protein. Total protein intake has not changed much during the past thirty to forty years, but the proportion of animal protein has increased from 30% to more than 50%. This change also contributed to the net increase in animal fat intake. In consequence, blood cholesterol levels in Japan have gradually increased, although the dietary cholesterol intake is lower on average than in Western countries. However, because many adults have a cholesterol phobia, the new RDA stresses limited intake, less than 300 mg of cholesterol daily, only for those who are prone to respond to dietary cholesterol with increases in their plasma cholesterol.

It may be concluded that more evidence is needed that supports the physiological functions of different types and amounts of dietary fats in "healthy people." Unfortunately, the current information mainly stems from their effects on those who are patients with chronic diseases, such as atherosclerosis. Although RDA for dietary fat in Japan seems at least to meet our health needs, we have many problems relating to our food supply. These problems are internal as well as external, and must be addressed in the interests of national public health. Further work is needed to see whether similar recommendations would also be optimal for other populations with a different cultural and genetic background.

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Fats in Indian Diets and Their Nutritional and Health Implications

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ABSTRACT: To arrive at fat requirements for Indians, the contribution of invisible fat should be determined. Total lipids were extracted from common Indian foods, and their fatty acid compositions were determined. This data and information on intake of various foods were used to estimate the contents of "invisible" fat and fatty acids in Indian diets. Taking into account World Health Organization (WHO) guidelines and the invisible-fat intake of Indians, recommendations were made for lower and upper limits of visible fats. In the rural poor, the "visible"-fat intakes are much lower than estimated minimum requirements. Therefore, to meet the energy needs of low-income groups, particularly young children, visible-fat intakes must be increased to recommended levels. The urban high-income group, however, should reduce dietary fat. Data on intake of various fatty acids in total diet shows that even the recommended lower limit of oil can meet linoleic acid requirements. Intake of α-linolenic acid is low, however. Increase in dietary n-3 polyunsaturated fatty acid (PUFA) produces hypolipidemic, anti-inflammatory, and antithrombotic effects. Effects of n-3 PUFA on blood lipids, platelet fatty acid composition, and platelet aggregation were therefore investigated in Indian subjects consuming cereal-based diets. Supplementation of fish oils (long-chain n-3 PUFA) as well as the use of rapeseed oil (α-linolenic acid) produced beneficial effects. Since the requirements of α-linolenic acid and/or long-chain n-3 PUFA are related to linoleic acid intake, use of more than one oil (correct choice) is recommended for providing a balanced intake of various fatty acids. Analysis of Indian food showed that some foods are good sources of α-linolenic acid. Regular consumption of these foods can also improve the quality of fat in Indian diets. Nonvegetarians, however, have the choice of eating fish to accomplish this.

Lipids 31, S-287–S-291 (1996).

In populations of developed countries, high fat consumption contributes to excess energy intake, and this is considered to be a risk factor for obesity and cardiovascular disease (1). The major nutrition related problem in India, however, is chronic energy deficiency associated with low fat intake (2). As the country is experiencing economic development, the urban affluent and middle-income groups are increasing in number, and there is a correspondingly steep escalation in the prevalence of obesity, diabetes, and cardiovascular disease in this section of the population (3). Since fat intake in India is income dependent and highly skewed (4,5), we focused our attention on the contribution of fat to nutritional status. This article encapsulates our work on contents of "invisible" fat and component fatty acids in Indian foods, offers insights into existing fat intake by Indians, and gives guidelines for ensuring correct quantity and quality of dietary fat for prevention of chronic energy deficiency on the one end and obesity and cardiovascular disease at the other end of the socioeconomic spectrum.

Dietary sources of fat are of two kinds: one is the "invisible" fat (i.e., fat present as an integral part of a food item) existing in different components of the habitual Indian diet, and the other is visible, or added, fat.

Invisible fat. Invisible fat in diet is derived from both animal and plant foods. Fat extracted by ether represents "free" lipids (6,7). Figures obtained with ether extraction show that plant foods have very little fat (8), and therefore it is not accounted for in terms of contributing to total fat intake. Since ether does not extract bound and structural lipids, it underestimates fat content in plant foods (6–8). To determine total invisible-fat content and its component fatty acids in Indian foods, food material was subjected to acid hydrolysis; total lipids (free + bound + structural) were extracted with a chloroform/methanol (2:1, vol/vol) mixture, converted to fatty acid methyl esters, and analyzed by gas chromatography against an internal standard (9,10). These data are summarized in Table 1. Although cereal and millet contain only 1–3% invisible fat, their status as bulk items in Indian diets contribute significantly to total fat intake. In cereal and millet, linoleic acid (LA) (18:2n-6) is the major fatty acid. On average, pulses and legumes provide more α-linolenic acid (ALNA) (18:3n-3) than do cereal and millet. Black gram, rajmah, and cowpea are rich sources of ALNA (LA/ALNA < 1.8). Commonly consumed condiments and spices contain significant amounts of fat. Both fenugreek and mustard seeds are rich sources of ALNA (LA/ALNA < 1.8). Analysis of fresh

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Abbreviations: ALNA, α-linolenic acid; CHD, coronary heart disease; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; LC, long chain; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

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vegetables showed that, on average, green leafy vegetables provide seven times more ALNA than other vegetables (LA/ALNA < 1.8) (10).

Visible fat. Several factors determine fat intake in the Indian population. First, fat intake is income dependent, and there are regional preferences in both the quantity and type of fat consumed (4,5,11). Vegetable oil is the major type of visible fat (national average 80%); vanaspati and ghee are other sources (12). Although there are several kinds of vegetable oil in India, groundnut and mustard/rapeseed oils make up 60% of the total production of oil (13).

As mentioned, there are regional preferences in the choice of oils. Groundnut oil is preferred in western and southern states. Sesame oil is preferred in south India, but in Kerala coconut oil is popular. Northern and eastern states use mustard oil. In north Karnataka and south Maharashtra, safflower oil is preferred. In recent years sunflower and rice bran oils have been introduced. Although imported palm oil has been used over the last decade, because of palm cultivation launched in India, indigenous palm oil is soon likely to be an important edible oil (13). The cost and advertisements claiming cholesterol-lowering potential of oil seem to influence the traditional and regional choice of oils, however. Vanaspati consumption is more common in northern states (12). Purchased ghee, as well as that made at home from milk, contributes to its high consumption in the urban affluent and middle-income groups (14). Relative proportions of fatty acids are known to vary in amounts of different visible fats.

Fat intake in different income groups. Dietary data from national surveys of the rural poor (an estimated 80% of the population) show that the average daily intake of cereal and millet is about 500 g, while visible-fat (oil) intake is below 10 g per person (2,4). Using data on invisible fat and fatty acids in Indian food (9,10) and information on dietary intake of the rural population (4), it has been found that cereal and millet alone furnish about 70% of the total invisible fat in the diet (9). Pulses, milk, fresh coconut, whole oilseed, and spices constitute the rest. The daily intake of cereal, millet, pulses, and

TABLE 1
Invisible Fat and Fatty Acids in Indian Foods (g/100 g food)[a]

Food	Botanical Name	Fat	16:0	18:0	18:1	18:2n-6	18:3n-3	18:2n-6/18:3n-3
Cereals and millets								LA/ALNA
Rice	*Oryza sativa*	1.7	0.40	0.05	0.4	0.5	0.01	41
Wheat	*Triticum aestivum*	2.9	0.40	0.10	0.3	1.1	0.17	6
Maize	*Zea mays*	4.8	0.70	0.20	1.1	2.2	0.05	47
Jowar	*Sorghum vulgare*	3.3	0.50	0.07	1.0	1.5	0.05	32
Ragi	*Eleusine coracana*	1.5	0.30	0.02	0.7	0.3	0.05	5
Bajra	*Pennisetum typhoideum*	5.5	1.00	0.24	1.2	2.2	0.13	17
Legumes/pulses								
Black gram	*Phaseolus mungo* Roxb	1.7	0.20	0.05	0.20	0.1	0.70	0.2
Rajmah	*P. vulgaris*	2.2	0.30	0.04	0.20	0.5	0.70	0.7
Cowpea	*Vigna catjang*	2.9	0.60	0.10	0.20	0.8	0.50	1.7
Green gram	*P. aureus* Roxb	1.7	0.40	0.05	0.05	0.6	0.20	3.0
Red gram	*Cajanus cajan*	2.2	0.40	0.08	0.10	1.0	0.10	8.0
Lentil	*Lens esculenta*	2.0	0.20	0.04	0.40	0.8	0.16	5.0
Bengal gram	*Cicer arietinum*	6.9	0.50	0.07	1.20	3.5	0.20	17.0
Peas	*Pisum sativum*	2.1	0.20	0.06	0.40	0.8	0.15	5.0
Soybeans	*Glycine max* Merr	20.0	1.40	0.80	5.40	10.4	1.40	7.0
Vegetables								
Fresh beans[b]		0.2	0.04	0.007	0.009	0.07	0.04	2.0
Green leafy vegetables[c]		0.4	0.05	0.007	0.025	0.04	0.15	0.3
Other vegetables[d]		0.2	0.03	0.008	0.016	0.06	0.03	2.0
Potato	*Solanum tuberosum*	0.6	0.15	0.002	0.150	0.08	0.06	5.0
Condiments and spices								
Dry chilies	*Capsicum annuum*	17	2.20	0.34	1.8	9.1	0.26	35.0
Cumin seed	*Caminum cyminum*	9	0.30	0.08	4.7	2.1	0.48	4.0
Coriander seed	*Coriandrum sativum*	20	0.60	0.10	12.5	3.0	0.02	129.0
Fenugreek seed	*Trigonella foenumgraecum*	10	0.90	0.30	1.5	3.4	1.90	1.8
Nuts and oilseeds								
Coconut	*Cocos nucifera*	40	8.0	4.0	3.2	0.6	—	—
Groundnut	*Arachis hypogaea*	40	3.5	1.7	21.0	10.0	0.2	50.0
Sesame	*Sesamum indicum*	40	4.0	1.6	18.0	16.0	0.4	40.0
Mustard	*Brassica nigra*	40	0.4	0.2	5.0	5.0	3.5	1.4

[a]Values are mean of three pooled market samples.
[b]Mean of three different types.
[c]Mean of fourteen different types.
[d]Means of five different types.

milk on an average provide about 16 g (7 en%) (en% = energy percent) fat, 5 g (2 en%) LA, and 0.6 g (0.2 en%) ALNA (9). Thus invisible fat from cereal and pulses alone furnishes two-thirds of the LA requirement (15). Two-thirds of fat present in cereal and millet are in the bound and structural forms (9). Studies in rats, however, have demonstrated that bound and structural fats in rice are biologically available (15). Diets of urban high-income groups are low in cereal (200–300 g/person/day) but contain more legumes, pulses, vegetables, and milk (also eggs and animal foods, in the case of nonvegetarians) (2,5). Therefore invisible-fat contribution to the diet is about 30 g (12 en%) (8). Average daily visible-fat intake in different segments of the urban population is about 13 g in slum-dwellers, 23 g in industrial laborers and low-income groups, 35 g in middle-income groups, and 46 g in high-income groups, but it is much higher in the top, affluent group (5,16).

Recommendation for visible fat in Indians. For adults, the World Health Organization (WHO) (17) proposed that the average intake for total fat should be between 15 and 30 en%. Taking into account the total invisible fat in diets of the rural population (9), the lower limit of visible fat (oil) in Indian diets is estimated to be about 20 g/person/day (9). Since chronic energy deficiency is a major nutritional problem in low-income groups, increasing fat/oil intake can help meet their energy needs.

Considering the amount of invisible fat in the urban high-income group, a daily intake of about 50 g/person (18 en%) would be the upper limit of visible fat (e.g., oil, ghee, vanaspati, and butter) (9). Because of the prevalance of obesity and coronary heart disease (CHD) in the urban population (3), their fat intake should be reduced.

In fulfilling the energy needs of young children through cereal/pulse diets, too much bulk would be a problem. To overcome this, it has been suggested that their diets should contain a minimum of 25 en% fat. This works out to be a daily intake of about 25 g (15 en%) per child (9). Widespread dietary energy deficiency in young children of low-income groups has been attributed to inadequate intake of diet (18). Re-examination of dietary energy intake and corresponding fat intake by preschool children belonging to different socioeconomic groups in both rural and urban areas, however, showed that increased energy intake is closely associated with increase in visible-fat intake (19). The data also show that, at visible-fat intakes corresponding to 15 en%, adequacy of energy intake is reached (20). These observations reinforce the importance of increasing energy density in diets of young children.

Quality of total fat with any visible fat. Several recommendations regarding quality of fat (21) have emphasized that the polyunsaturated fatty acid/saturated fatty acid (PUFA/SFA) ratio should be between 0.8 and 1.0. WHO (18) proposed that the range for SFA be between 0 and 10 en% and that for PUFA it should be between 3 and 7 en%. Currently, in view of the varied roles of individual SFA and PUFA, the PUFA/SFA ratio is considered obsolete, and lower and upper limits of the safe range of intake of various unsaturated fatty acids have been suggested. A balance in the intake of LA and ALNA is essential for maintaining a balance in the formation of eicosanoids of "2" and "3" series, respectively. It is therefore recommended that the dietary LA/ALNA ratio should be kept between 5 and 10. Dietary LA/ALNA ratios above 50 lead to tissue depletion of decosahexaenoic acid (DHA) and are therefore regarded as indicative of ALNA deficiency.

Quantity and quality of both invisible and visible fats determine the intake of various fatty acids in the total diet. In diets of the rural poor, invisible fat furnishes about 5 g SFA, 4 g monounsaturated fatty acid (MUFA), and 0.3 g ALNA, and the ratios of PUFA/SFA and LA/ALNA are about 1 and 13, respectively. Assuming that 20 g of any one fat/oil (suggested lower limit) is the source of visible fat, the probable fatty acid intake in the total diet was calculated (Table 2). It was found that, with any one oil, although minimum LA requirements may be met, ALNA intakes are only adequate when mustard/rapeseed or soybean oil are used. Therefore, with oils other than these, the LA/ALNA ratios are very high.

In the urban high-income group, invisible fat furnishes about 13 g SFA, 6 g MUFA, 4 g LA, and 0.6 g ALNA, and the ratios of PUFA/SFA and LA/ALNA are 0.3 and 6.0 respectively. Taking this into account, probable fatty acid intake in the total diet of this socioeconomic group is calculated with 50 g (suggested upper limit) of any fat/oil (Table 3). It was found that use of any one oil furnishes SFA about or above the upper limits of the safe range. Use of oil like safflower, sunflower, sesame, and soybean furnish high levels of LA. ALNA levels are in the desirable range only when mustard/rapeseed, soybean, or rice bran oils are used. Mustard/rapeseed oils provide about 17 en% MUFA, 13 en% of which are long chain (LC) MUFA (20:1 and 22:1). Use of safflower, sunflower, sesame, or groundnut oil as a single source of visible fat provides very high ratios of LA/ALNA. It therefore appears that, in both low and high socioeconomic groups, as long as the LA requirements are fully met (with all oils except coconut), there is a

TABLE 2
Probable Daily Intake of Fatty Acids in Total Diet of Rural Poor with 20 g Oil[a]

Oil	SFA[b] en% (g)	MUFA[c] en% (g)	LA[d] en% (g)	ALNA[e] en% (g)	PUFA[f]/ SFA	LA/ ALNA
Safflower	2 (6)	3 (7)	9 (20)	0.1 (0.3)	3.0	54
Sunflower	3 (7)	3 (8)	8 (18)	0.2 (0.4)	3.0	46
Sesame	3 (8)	4 (10)	6 (14)	0.2 (0.4)	2.0	36
Rice bran	3 (8)	5 (11)	4 (10)	0.3 (0.6)	1.3	17
Groundnut	4 (9)	5 (12)	5 (12)	0.2 (0.4)	1.0	30
Palm	6 (13)	6 (13)	3 (7)	0.2 (0.4)	0.6	16
Coconut	10[g] (22)	2 (5)	2[h] (5)	0.1 (0.3)	0.2	15
Mustard/ rapeseed	2 (6)	8[i] (18)	4 (9)	1.5 (3.4)	2.0	3
Soybean	3 (7)	4 (9)	7 (16)	0.6 (1.3)	2.0	12
Safe range						
en%	0–10	0–20	3–10	0.5–2.5	0.8–1.0	5–10
g	0–26	0–51	8–26	1–6		

[a]Suggested lower limit.
[b]SFA = saturated fatty acid.
[c]MUFA = monounsaturated fatty acid.
[d]LA = linoleic acid.
[e]ALNA = α-linolenic acid.
[f]PUFA = polyunsaturated fatty acid.
[g]≤14:0, 7 en%.
[h]Basic requirements not met.
[i]Long-chain MUFA, 6 en%.

need to increase the intake of ALNA and/or LC n-3 PUFA. In the low socioeconomic group, there is scope to increase SFA intake. It is suggested that an increase in milk intake would not only raise SFA levels but would also provide several other nutritional benefits.

PUFA nutritional status from phospholipid fatty acid composition. Dietary unsaturated fatty acids cause changes in the fatty acid composition of membrane phospholipids and in the subsequent eicosanoid spectrum (21). Enrichment of membranes with LC n-3 PUFA [eicosapentaenoic acid (EPA) and DHA] produces antithrombotic and anti-inflammatory effects (21). An inverse relationship has been reported between death rates from CHD and the ratio of arachidonic/eicosapentaenoic acids (AA/EPA) in plasma and platelet phospholipids. In populations with high death rates due to CHD (United States and Europe), the AA/EPA ratio in platelet phospholipids is about 50, whereas in the Japanese and Eskimo populations, which are less susceptible to CHD, this ratio is about 12 and 1.2, respectively (22). The fatty acid composition of platelet phospholipids was determined in apparently normal men (n = 70) belonging to an urban high-income group (Ghafoorunissa, unpublished). Wide variation was observed in the levels of EPA (1.2 ± 0.14) and AA (20 ± 0.6). EPA was not detected in 25 subjects. Mean ratio of AA/EPA was 56 ± 12 (n = 45). In about one-third of the subjects studied the mean ratio was 153 ± 24.

Effects of oils rich in n-3 PUFA on blood lipids and platelet fatty acid composition. The n-3 PUFA have hypolipidemic, anti-thrombotic and anti-inflammatory effects (21). Therefore, for prevention of several chronic degenerative diseases including CHD, increase in the intake of LC n-3 PUFA (fish and fish oil) and/or ALNA (plant foods) are recommended. From the previous discussion, it is clear that the quality of fat in Indian diets can be improved by increasing n-3 PUFA intake. Studies were therefore conducted (23) in men consuming cereal-pulse–based diets providing about 26 en% fat to determine the efficacy of using ALNA in comparison to LC n-3 PUFA for producing hypolipidemic and antithrombotic effects. Keeping LA levels constant, n-3 PUFA levels were altered by supplementing 0.2 or 0.5 en% LC n-3 PUFA (MaxEPA). To increase dietary ALNA, the visible-fat source was changed from groundnut oil (ALNA negligible) to zero erucic acid rapeseed oil (ZEAR) (ALNA 9%). The ratios of n-6/n-3 PUFA, initially 31, were reduced to 12 and 8, respectively, with LC n-3 PUFA, and to 4 with ZEAR. Results showed a decrease in total cholesterol with both levels of LC n-3 PUFA, but triglycerides were reduced with only the higher dose. Dietary increase in LC n-3 PUFA as well as in ALNA increased levels of LC n-3 PUFA in both plasma and platelet phospholipids and reduced collagen-induced platelet aggregation. The magnitude of changes were less marked with ALNA, as compared to LC n-3 PUFA, however. These observations indicate that it may be possible to favorably modify platelet functions by reducing the dietary LA/ALNA ratio. Other researchers have reported similar changes (24,25).

Mustard oil produced in India has high levels of LC MUFA (20:1 and 22:1). Extensive research has documented that LC MUFA present in mustard and rapeseed oils produce undesirable effects in experimental animals (26). Therefore, Sweden and Canada grow low–erucic-acid varieties. It has been shown that human hearts obtained during accident autopsies in Calcutta (where mustard oil is used as a major source of visible fat) have high levels of erucic acid (27).

Guidelines to ensure quality of fat. The studies discussed herein have demonstrated beneficial effects of increasing dietary n-3 PUFA (ALNA or LC n-3 PUFA) at a fixed intake of LA. Since requirements of n-3 PUFA increase with an increase in n-6 PUFA intake, it is important to moderate LA (and LC n-6 PUFA) and to increase ALNA and/or LC n-3 PUFA intake. To ensure this in the existing dietary patterns of Indians, it is advisable to (i) use oil with moderate levels of LA such as groundnut, rice bran, or sesame; (ii) when oil with high LA (safflower, sunflower, cottonseed, and soybean) is used, it can be utilized in conjunction with those that contain low LA (e.g., palm oil); (iii) use of an oil of choice along with mustard oil in approximately equal proportions will not only increase ALNA but will also keep the LC MUFA intake from mustard oil low; (iv) it is interesting to note that the combination of soybean oil with palm oil in equal proportions can also ensure quality of fat in Indian diets; (v) in addition to the constituent fatty acids, minor components present in fat/oil contribute to their nutritional and health effects. Some of these are powerful natural antioxidants (28). Use of more than one source of oil gives the added advantage of providing a greater variety of these minor components in the diet; (vi) regular consumption of ALNA-rich foods (Table 1) can contribute to increased dietary ALNA. Use of green leafy vegetables every day will not only increase ALNA intake but will also provide several other nutritional benefits (8,18); (vii) to improve the quality of fat, nonvegetarians should eat fish. Analysis of commonly consumed fish (29) indicate that fish with high fat (>5 g/100 g), medium fat (1–5 g/100 g), and low fat

TABLE 3
Probable Daily Intake of Fatty Acids in Total Diet of Urban High-Income Groups with 50 g Oil[a]

Oil	SFA en% (g)	MUFA en% (g)	LA en% (g)	ALNA en% (g)	PUFA/ SFA	LA/ ANLA
Safflower	7 (17)	6 (14)	17 (42)	0.2 (0.6)	2.4	69
Sunflower	8 (19)	7 (17)	15 (37)	0.3 (0.7)	1.9	57
Sesame	9 (23)	9 (22)	12 (29)	0.3 (0.7)	1.3	41
Rice bran	9 (23)	10 (24)	8 (18)	0.5 (1.2)	0.9	15
Groundnut	10 (25)	11 (27)	9 (21)	0.3 (0.7)	0.9	32
Palm	14 (34)	12 (30)	4 (10)	0.3 (0.7)	0.3	13
Mustard/ rapeseed	7 (16)	17[b] (42)	4 (10)	1.8 (4.0)	0.9	2
Soybean	8 (20)	8 (20)	13 (32)	1.2 (3.0)	1.8	10
Coconut	24[c] (58)	4 (10)	2[d] (5.0)	0.2 (0.6)	0.1	9
Safe range						
en%	0–10	0–20	3–10	0.5–2.5	0.8–1.0	5–10
g	0–26	0–51	8–26	1–6	—	—

[a]Upper limits; see Table 2 for abbreviations.
[b]Long-chain MUFA, 13 en%.
[c]≤14:0, 16 en%.
[d]Basic requirements not met.

(<1 g/100 g) furnish an average of about 1.2, 0.4, and 0.1 g LC n-3 PUFA per 100 g muscle, respectively.

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Impact of Fat Substitutes on Fat Intake

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ABSTRACT: Dietary fat is the number one nutrition concern of Americans. In response to rising consumer demand for reduced-fat foods, the food industry has developed a multitude of nonfat, lowfat, and reduced-fat versions of regular food products. To generate reduced-fat or fat-free products that have the same organoleptic characteristics of the regular fat version, food manufacturers frequently employ fat substitutes in the formulation of these foods. Fat substitutes are made from either carbohydrate, protein, or fat, or a combination of these components. Researchers have questioned the impact of fat substitutes on both fat and caloric intake. The majority of research studies in which fat substitutes were either covertly or overtly substituted for dietary fat indicate that in short-term, carefully-controlled conditions, fat substitutes can decrease both dietary fat intake and percentage of calorie intake from fat. However, individuals compensate for the caloric deficit created by the fat substitutes by increasing their consumption of other macronutrients, primarily carbohydrate. The long-term effect of fat substitutes on the fat intake of free-living individuals and weight control are unknown. People tend to eat more of a food when they know that food is reduced in fat. Fat substitutes should not be considered a substitute for sound nutrition education and a healthy lifestyle which includes regular exercise.
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Today's Americans are fat-phobic. Dietary fat is the number-one nutrition concern of Americans (1). Nearly three-quarters of adults have changed their diets to decrease their fat intake (2). Three out of every four adults report that they consume reduced-fat foods and beverages (3). And just over one-half (54%) of U.S. adults choose reduced-fat versions of snack foods over the full-fat versions (4). In response to rising consumer demand for reduced-fat foods, the food industry has developed a multitude of nonfat, lowfat, and reduced-fat versions of regular food products. Today, roughly 14% of all new foods introduced into the marketplace are either reduced fat, reduced cholesterol, or reduced calorie (5).

New food products containing fat substitutes include hot dogs, bologna, oven-roasted chicken breast, smoked turkey breast, cheese, baked goods, frozen dessert, mayonnaise, salad dressing, sour cream, and potato chips (6). To generate reduced-fat or fat-free products that have the same organoleptic characteristics of the regular-fat versions, food manufacturers frequently employ fat substitutes in the formulation of these foods.

Dietary fat substitutes have existed for over a decade (7), and over the past ten years, more than 100 fat substitutes have been formulated (8). Fat substitutes are made from carbohydrate, protein, fat, or a combination of these components. Carbohydrate fat substitutes include gums such as xanthan gum; algins from kelp; modified food starches such as Stellar and STA-SLIM 143 (A.E. Staley Mfg. Co., Decatur, IL); dextrins; polydextrose; maltodextrins such as Matrin MO40, N-Oil, and Paselli SA_2 (Avebe America, Princeton, NJ); mixtures of dextrins and fiber such as Nutrifat®; fiber-based products such as inulin and oatrim (Rhone-Poulenc, Inc., Princeton, NJ); cellulose-based products such as Avicel (FMC Corp., Philadelphia, PA); and pectin-based products such as Slendid (Hercules Food Gums, Wilmington, DE) (9,10). Carbohydrate-based fat substitutes absorb water and imitate the thickness and creaminess of fat. These products are used in baked products, pudding, salad dressing, yogurt, milk, cheese products, processed meat products, spreads, dips, frosting, soup, and frozen dessert. Carbohydrate-based fat substitutes typically provide between 1 and 4 calories per g and are limited by the fact that they are often not well suited to baking, cannot be used in frying, and may reduce shelf life due to a high associated water content (21).

Protein-based fat substitutes include Simplesse® (Simplesse Co., Deerfield, IL) and Trailblazer (Kraft General Foods, Glenview, IL) (22–24). Simplesse® is made through the application of a patented heating and blending process (called microparticulation) to milk or egg-white protein. Trailblazer is created from a mixture of egg white, whey protein, and xantham gum. These fat substitutes are used in cheese, ice cream, yogurt, mayonnaise, dressing, spreads, cake, muffins, pastries, frosting, and frozen cheesecake. Like carbohydrate-based fat substitutes, protein-based fat substitutes provide between 1 and 4 calories per g and are limited by the fact that they are not well suited to baking and cannot be used in frying (21).

Fat-based fat substitutes include sucrose polyester [better known as olestra (Procter & Gamble Co., Cincinnati, OH)],

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Abbreviation: GRAS, generally regarded as safe.

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salatrim (Pfizer, Inc., New York), sorbestrin, caprenin (Procter & Gamble, Cincinnati, OH), appetize (Omegasource Corp., Burnsville, MN), and lita®. Salatrim, sorbestrin (Pfizer, Inc., New York), and caprenin provide between 5 and 9 calories per g, whereas olestra is a nondigestible compound (25–28). Fat-based fat substitutes may be used to replace fat in products such as cheese, peanut butter, potato chips, candy, spreads, oil, and ice cream.

The ease with which a fat substitute is introduced into the marketplace depends on its method of formulation. New molecules such as olestra must first be cleared with the Food and Drug Administration (FDA) as a food additive before they are used in food products. Procter and Gamble has been seeking FDA approval of the use of olestra in snack foods for over eight years. In support of this product, Procter and Gamble conducted 100 animal studies and 25 clinical trials on olestra, accumulated over 100,000 pages of research data, and spent over $200 million (26,29–31). The FDA has currently granted Procter and Gamble an extension for its patent, which expires in 1996.

If the development of a fat substitute involves applying a new process to an existing molecule (such as utilizing microparticulation to generate Simplesse), the FDA may require manufacturers to submit a petition to affirm that the fat substitute is generally regarded as safe (GRAS) (25). If, instead, the formulation of a fat substitute involves applying existing technologies to familiar molecules (such as the manufacture of maltodextrin), the fat substitute will be more readily incorporated into the marketplace (18,32).

The optimal fat substitute should feel, taste, and act like real fat when cooked or fried and contain little or no calories. Unfortunately, success in meeting all of these criteria has been limited (8,33). Most fat substitutes simulate the texture but not the taste or melting properties of real fat (26). Most aromatic chemicals that influence flavor are soluble in fat. Consequently, carbohydrate- and protein-based fat substitutes are limited by the fact that they cannot carry these compounds (26,34).

Taste is probably the most pleasing characteristic of dietary fat. Taste is generally the number-one reason why people select foods. People find it difficult to reduce their fat intake primarily because fat greatly enhances the flavor of foods (35). Reduction in the taste quality of the diet is the reason most frequently cited for consumers' inability to adhere to a reduced-fat diet (36). Even if fat substitutes can be formulated to taste just like real fat, however, the scientific community has questioned whether fat substitues will provide a significant health benefit to consumers. Researchers have raised concerns that (i) unlike dietary fat, fat substitutes do not provide essential fatty acids and fat soluble vitamins; (ii) if poorly absorbed, fat substitutes may absorb nutrients and drugs such as oral contraceptives, preventing their absorption; and (iii) fat substitutes may adversely alter the function of the gastrointestinal tract (25,26).

Additionally, researchers have questioned the impact of fat substitutes on both fat and caloric intakes. According to one estimate, dietary fat intake could be reduced by up to 10 g per day if fat-free alternatives were substituted for regular-fat cream cheese, cottage cheese, cheesecake, sour cream, salad dressing, frozen desserts, and sweet baked goods (37,38). Considering the reduction in calories that would also assumably occur when fat-free alternatives are substituted for traditional fat-containing foods, researchers estimated that use of these fat-free products would reduce the percent of total calorie intake from fat from 36 to 31%. Similarly, it has been estimated that use of Simplesse in all potential applications could reduce total fat intake from 36 to 30% of all calories (39).

The majority of controlled studies conducted on either lean or obese subjects indicate that fat substitutes can decrease dietary fat intake (35,40–44). These studies reduced intake by replacing the fat in muffins, biscuits, potato chips, cookies, frozen desserts, or shakes. One study conducted in obese adults replaced 60 g fat in a shake with olestra (41). This study reduced the daily percent of calorie intake from fat from a control level of 43 to 29% (41). A more recent study conducted in lean men replaced either 20 or 36 g fat in breakfast biscuits with olestra (35). The control diet provided 42% of total calories from fat. Replacing 20 g fat with olestra reduced the daily percent of calorie intake from fat to 39%. Replacing 36 g fat in biscuits with olestra further reduced the daily percent of calorie intake from fat to 35% (35). A 1993 study conducted in children replaced 25 g fat in a frozen dessert with Simplesse (43). This study achieved only a 1% reduction in calorie intake from fat (i.e., reducing daily fat intake from 37 to 36% of calories). Consequently, under very controlled research conditions, replacing dietary fat with fat substitutes can decrease caloric fat intake by as much as 14%. Keep in mind, however, that these studies determine the fat content of the control as well as the test diets. The length of these studies ranged from 8 to 40 days. Therefore long-term effects of fat substitutes on dietary fat intake are unknown.

In a free-living situation, fat substitutes may not produce dramatic decreases in fat intake. Using a computer modeling system, researchers have determined that several different dietary strategies are needed to realistically reduce fat intake (45,46). No single strategy can be expected to reduce fat intake to the goal of 30% of calories. Furthermore fat-reduction strategies that work best for specific people are highly individualistic (46). Consumers should be given a choice of many different fat-reduction strategies and allowed flexibility to choose strategies that best meet their needs (46). In addition, most studies conducted with fat substitutes report that individuals compensate for the caloric deficit created by use of the fat substitute by increasing their intake of carbohydrate and protein (35,42,43). This energy compensation is both unconscious (as demonstrated by studies that covertly replace dietary fat with fat substitutes (35,42,43) and conscious, as demonstrated by studies where the subjects are told they are consuming a reduced-fat food (47). People tend to eat more of a food when they know that the food is reduced in fat (47–50). Consequently, foods containing fat substitutes may help consumers reduce their dietary fat intake but not necessarily their

caloric intake, because individuals may compensate by consuming more food.

Dietary fat intake in the United States has been falling since the 1960s (51). At present, Americans consume approximately 34% of their calories from fat (62). In contrast, calorie intake and the rate of weight gain and obesity has been increasing (53,54). Today, one in three Americans is overweight (1). The current prevalence of overweight people in the United States, despite years of weight-reduction efforts, is hardly encouraging (5). Almost no data exist on the effect of fat substitutes on weight loss (6). To lose weight, it is equally important that consumers decrease their calorie intakes and increase their activity levels (57,58). Unfortunately, today, only about 7% of Americans are concerned about calories, whereas 60% are concerned about fat (1).

Will fat substitutes improve the nutrient intake of Americans by alleviating consumers' ill-founded "guilt" over consuming nutrient-dense foods from the five food groups that also happen to contain fat? Or will fat substitutes drive consumers to eat a class of foods that are low in fat but also equally low in nutrients (21)? Currently available research does not allow us to make speculations at this point.

Health professionals are also divided over the possible effect of fat substitutes on taste preference. Whereas some speculate that good-tasting foods containing fat replacements may promote a gradual acquisition of a preference for lower-fat foods (31), others propose that foods containing fat substitutes may support a taste preference for high-fat foods (59). Research indicates that people in general, and overweight people specifically, prefer the taste of high-fat foods (60). Scientists have also raised questions about the inability of low-fat foods to provide long-term satiety, compared to high-fat products (61). Some researchers hypothesize that the influence of dietary fat on satiety impacts both the perception of palatability and food preference (21). Consequently, will fat substitutes heighten a taste preference for high-fat foods that will only be satisfied by the consumption of products high in real fat?

As health professionals, we must teach consumers that macronutrient substitution in foods is not a substitute for a healthy lifestyle, which includes consuming a nutritious diet, maintaining an appropriate weight, and exercising on a regular basis (5). Foods that contain fat substitutes can be a part of a healthy lifestyle but should not be promoted as a "magic bullet" to dietary fat reduction, weight loss, or good nutrition. Instructing people to maintain a healthy lifestyle, which includes eating more foods from the five food groups, consuming fewer desserts and sweets, and exercising on a regular basis may sound like less savvy advice than offering consumers fat-free potato chips and candy bars. In the long run, however, sound nutrition advice based on principles of the five food groups, variety, and moderation, together with encouragement of an active and healthy lifestyle, will achieve greater success in optimizing the nutritional status of Americans than any proposed magic bullet.

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Production of Eicosapentaenoic Acid from Marine Bacteria

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ABSTRACT: A marine bacterium, judged as a new species close to *Shewanella putrefaciens*, was isolated from the intestinal contents of the Pacific mackerel. The isolated strain SCRC-2378 produced eicosapentaenoic acid (EPA) as the sole polyunsaturated fatty acid, which amounted to 24–40% of the total fatty acid in the cell, which corresponded to 2% of dry cell weight. Under the optimal growth conditions (pH 7.0, 20°C, and grown aerobically for 12–18 h), the yield of SCRC-2738 reached 15 g of dry cells/L or 2×10^{10} viable cells/mL. EPA existed as phospholipid and was found in the *sn*-2 position of phosphatidylethanolamine and phosphatidylglycerol. The 38 kbp (1,000 base pairs) genome DNA fragment was cloned from SCRC-2738 and expressed in *Escherichia coli*, which resulted in the production of EPA. The nucleotide sequence of the 38 kbp DNA fragment was determined. The DNA fragment contains eight open reading frames, and three of them possess homology with enzymes involved in fatty acid synthesis. Thus, it may be possible that these EPA biosynthesis genes are applied for EPA production in yeasts or higher plants, and offer a new method for EPA synthesis as new foods containing EPA.
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Eicosapentaenoic acid (EPA) is an important component in the cell membranes of animals, and its pharmacological functions are well known (1). The principal bioactivities associated with EPA are summarized in Table 1. The pharmacological functions of EPA have generally been explained from the viewpoint of an antagonistic action against arachidonic acid (2–7), especially in the case of coronary vascular disease.

The origin of EPA has been explained by a series in the marine food chain as follows: microalgae → phytoplankton → zooplankton → small fish → larger fish and marine animals. It is generally accepted that the phytoplankton are the primary producers of EPA. However, it was thought EPA may also be produced by some of the symbiotic microorganisms which are present in the intestines of blue-back fish rich in EPA, in addition to being produced by the food chain series described previously. Thus, our search for EPA-producing bacteria started, and we succeeded in finding bacteria which produced EPA. We first cloned the EPA biosynthesis gene cluster from the EPA-producing bacteria, and its analysis is in progress.

Abbreviations: EPA, eicosapentaenoic acid; GLC, gas–liquid chromatography; kbp, 1,000 base pairs; ORF, open reading frame; PE, phosphatidylethanolamine; pEPA, the plasmid isolated from strain No. 95 of SCRC-2738; PG, phosphatidylglycerol.

TABLE 1
Distribution of Fatty Acids in Phospholipids in SCRC-2738

	Fatty acid composition (mol%)			
	Phosphatidylethanolamine		Phosphatidylglycerol	
Fatty acids	*sn*-1 (α)	*sn*-2 (β)	*sn*-1 (α)	*sn*-2 (β)
14:0	3.2	0.8	1.1	0.4
i-15:0[a]	11.5	7.2	4.5	2.1
15:0	0.5	0.3	3.4	0.4
16:0	12.1	0.6	10.7	1.6
16:1	6.8	20.9	8.0	11.5
i-17:0	0.6	0	0.9	0
17:0	1.8	0	2.7	0.3
17:1[b]	4.0	6.4	5.7	6.1
17:1[b]	1.6	0	2.2	0.3
18:0	0.4	0.1	0.6	0.3
18:1(9)	1.1	1.0	1.5	1.2
18:1(11)	6.1	0.7	7.8	1.1
20:5	0.1	11.8	1.0	24.7

[a]*iso*-Type fatty acid.
[b]Isomers in position of double bond.

METHODS

The intestinal contents of the marine animals were diluted with sterilized seawater and the diluted contents were cultured in dozens of agar media for the selection of bacterial colonies. Various conventional media supplemented with artificial seawater (Jamarin Laboratory, Osaka, Japan) were used (8,9). Colonies formed on various agar media were cultivated singly in the liquid media to test for the presence of EPA in the cellular lipid. Each EPA-positive strain was tested immediately for bacteriological purity. The promising strains were then subjected to tests involving about 60 items for classification and identification and, after testing, were subcultured and stored.

The lipids of bacterial cells were extracted by the Folch method (chloroform/methanol, 2:1, vol/vol). The total lipid extract was then saponified and the free fatty acids obtained were methylated with CH_2N_2 and analyzed by gas–liquid chromatography (GLC) equipped with a capillary column.

Chromosomal DNA was isolated from strain SCRC-2738, an EPA-producing bacteria. The DNA was partially digested with Sau3AI and fractionated by size on 0.3% agarose gel (Sigma, St. Louis, MO). The fragments longer than 20 kbp (kbp = 1,000 base pairs) were recovered by electroelution and then ligated with BamHI-digested and dephosphorylated cosmid pWE15. The ligated DNA were packaged into the phage heads and transfected into *Escherichia coli* AG1. The trans-

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formants were selected for expression of ampicillin resistance and cultivated singly in the liquid media to test for the presence of EPA in the cellular lipid.

The plasmid was isolated from an EPA-producing clone and its insert DNA fragment was subcloned for sequencing. The nucleotide sequence was determined by the dideoxy chain termination procedure.

RESULTS

About 50,000 bacterial strains were isolated from the intestines of various marine fish and other marine animals. Each of those strains was cultured as a single clone and was assessed for EPA levels by GLC analysis. EPA-producing bacteria were isolated with relatively high frequency from the blue-black fish containing high levels of EPA in their oils.

There were also several new species of marine bacteria which were phylogenetically similar to *Shewanella putrefaciens*. Among them, the intestinal strain SCRC-2738, isolated from a horse mackerel (9), had the highest EPA productivity. An electronmicrograph of SCRC-2738 (Fig. 1) indicates its size and presence of multiple flagella.

The fatty acid composition of the bacterium was simple and characteristic (10)—EPA was the only polyunsaturated fatty acid detected in significant quantities. In addition to EPA, several monoenoic acids and saturated fatty acids of the C_{13}–C_{18} range were detected. The percentage of EPA to the total fatty acids ranged from 25–40%, depending on the conditions of cultivation. In the fatty acid composition of SCRC-2738, almost none of the polyunsaturated fatty acids, varying from $C_{18:2}$ to $C_{20:4}$, were detected. This fact could be due to a high level of activity of the series of biosynthetic enzymes producing EPA.

We concluded that 90–95% of the EPA was in the form of phospholipids, and the free fatty acid fraction accounted for the remaining 5–10%. Furthermore, EPA was distributed almost equally between phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). The PE and PG were studied further to determine the positional specificity of the EPA within the molecules. The PE and PG fractionated by thin-layer chromatography were isolated and mixed with snake venom phospholipase. It was found that the EPA in both PE and PG was specifically bound by an ester bond to the *sn*-2 position (Table 1).

The SCRC-2738 was cultivated in a medium (half-strength, artificial seawater containing 1–3% peptone and

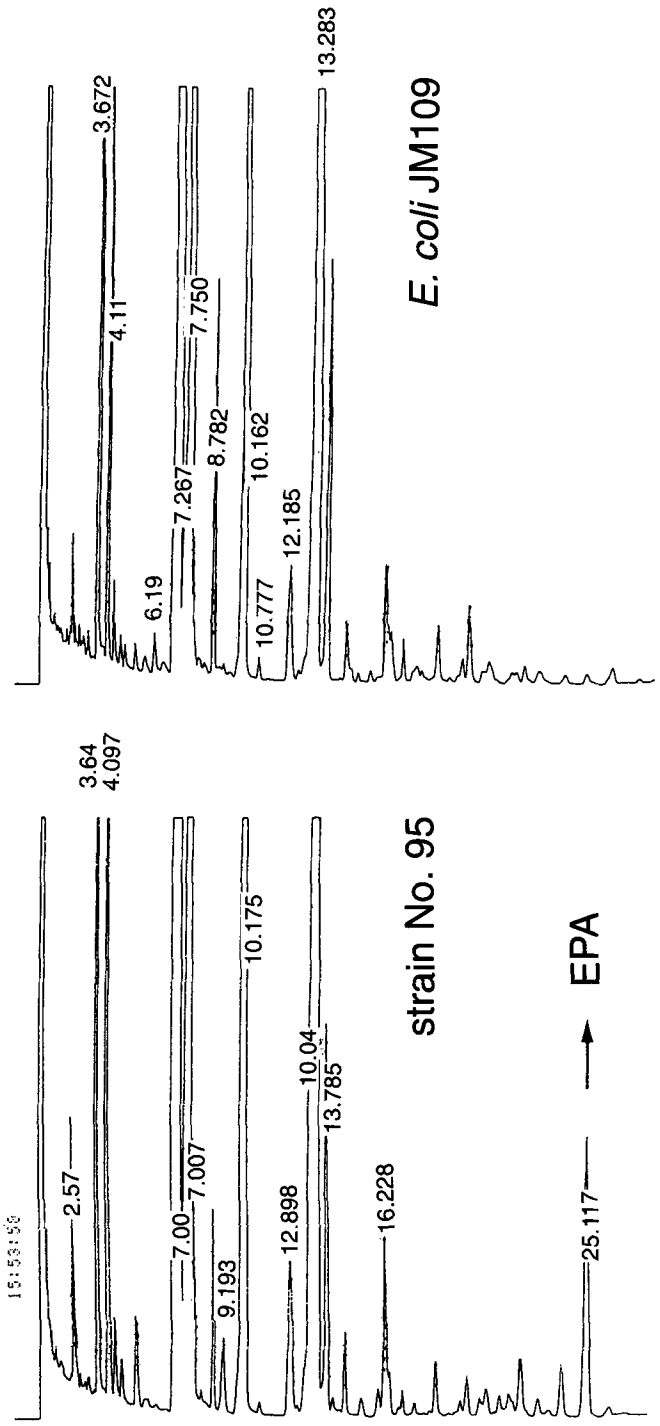


FIG. 1. Transmission electron micrograph of SCRC-2738 with negative staining.

FIG. 2. Gas–liquid chromatography of fatty acid methyl esters from the cells of strain No. 95 and *Escherichia coli* JM109. EPA, eicosapen-

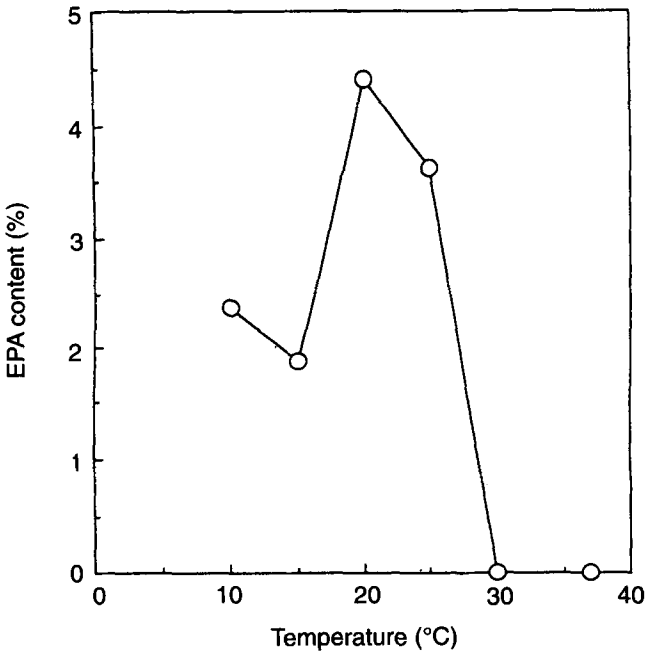


FIG. 3. Effect of growth temperature on EPA content in total fatty acids of strain No. 95. Abbreviation as in Figure 2.

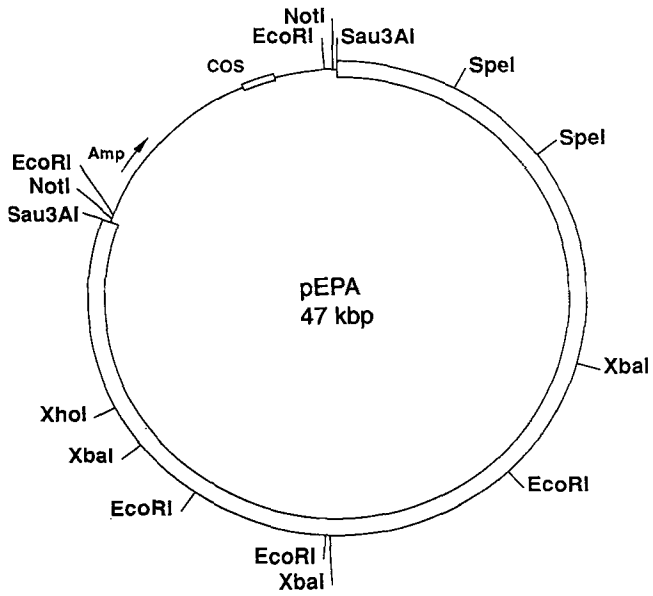


FIG. 4. Restriction map of plasmid pEPA. Abbreviation as in Figure 2.

0.5–1% yeast extract, pH 7.0) at 10 to 15°C with plenty of aeration. Under these conditions, 200–400 mg EPA was produced per liter of culture in 8 to 12 h (11). Furthermore, good EPA productivity was obtained when corn steep liquor or marine industrial waste liquid was used as a carbon source, thereby considerably reducing the overall fermentation costs. In this case, lactic acid was used to prevent the production of NH_3 and the increase of pH during cultivation. It may be possible to further reduce the cost of production by using a fed-batch culture, a high-density culture, and/or gene technology.

A genome DNA library of the SCRC-2738 was constructed and screened by EPA production. In a total of 390 analyzed clones, only strain No. 95 showed EPA productivity (Fig. 2). This means that strain No. 95 has the complete plasmid containing EPA biosynthesis genes.

It is known that growth temperature affects cellular EPA contents in SCRC-2378. As the cultivation temperature increased above 25°C, the productivity of EPA decreased, and no EPA production was observed at 30°C or higher temperature. The study of recombinant strain No. 95 indicated similar results (Fig. 3).

The plasmid was isolated from strain No. 95 and was designated pEPA; pEPA contains a 38 kbp genome DNA fragment from SCRC-2738 in the BamHI site of 8.8 kbp cosmid pWE15 (Fig. 4). The 38 kbp DNA fragment was subcloned and its nucleotide sequence was determined. In this fragment, eight open reading frames (ORF) larger than 1.0 kbp existed. To determine the ORF responsible for EPA synthesis, several deletion mutants of pEPA were constructed. pEPA-d1, which lacks XhoI-AscI fragment containing ORF1, indicates EPA

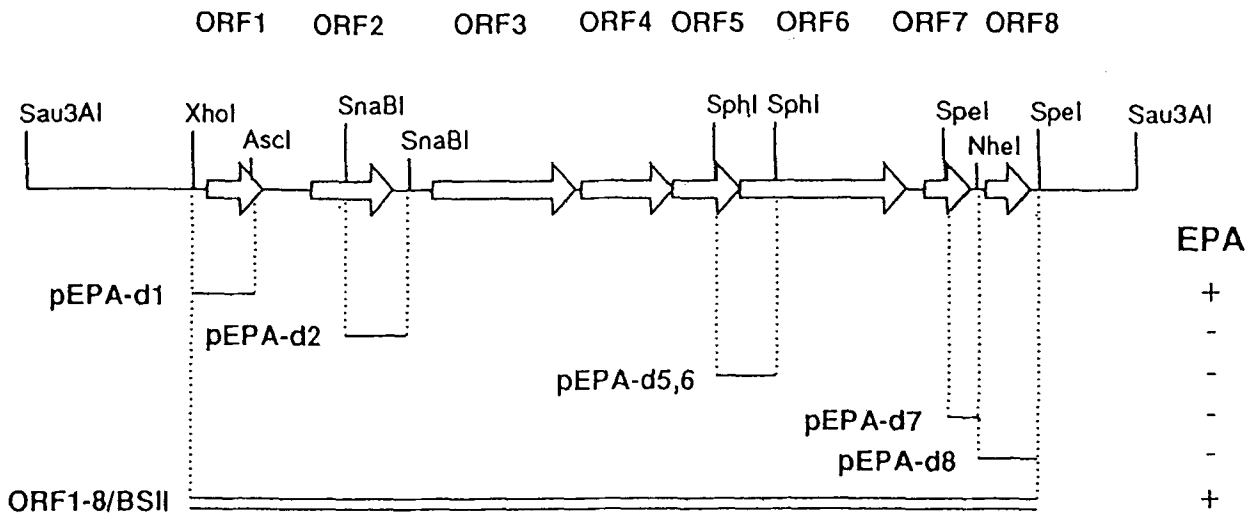


FIG. 5. EPA productivity of deletion mutants of pEPA. ORF, open reading frame; other abbreviation as in Figure 2.

TABLE 2
Homologous Enzymes with Open Reading Frames (ORF) of pEPA[a]

ORF no.	Enzymes (origin)
3	3-Oxoacyl-ACP synthase I (*Escherichia coli*) 26.9%/290aa
3	Malonyl CoA-ACP transacylase (*E. coli*) 29.1%/265aa
4	3-Oxoacyl-ACP reductase homologous protein (*Saccharopolyspora erythraea*) 30.5%/167aa
6	3-Oxoacyl-ACP synthase (Barley chloroplast) 29.1%/234aa
6	3-Hydroxydecanoyl-ACP dehydratase (*E. coli*) 31.0%/142aa

[a]pEPA (eicosapentaenoic acid) is the plasmid isolated from recombinant *E. coli* strain No. 95.

FIG. 6. Application of EPA biosynthesis genes for EPA production in yeast or plant. Abbreviation as in Figure 2.

productivity. However, pEPA-d2 (lacking ORF2), pEPA-d5,6 (lacking ORF5 and ORF6), pEPA-d7 (lacking ORF7), and pEPA-d8 (lacking ORF8) lost EPA productivity (Fig. 5). These results suggest that these ORF, except for ORF1, are involved in EPA synthesis. The deletion studies of other ORF are in progress.

A homology search of amino acid sequences deduced from the nucleotide sequences of the ORF with proteins in our database was done. ORF3, ORF4, and ORF6 showed a similarity with several enzymes involved in fatty acid synthesis (Table 2). This finding suggests that these ORF are involved in fatty acid *de novo* synthesis or in carbon chain elongation. Therefore, it is suggested that the ORF coding desaturase genes exist in the remaining ORF. The function of these ORF may be revealed by further studies. It may be possible that these EPA biosynthesis genes are applied for EPA production in yeast or plant, and offer a new method for polyunsaturated fatty acid synthesis (Fig. 6).

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Eicosapentaenoic Acid Suppressed the Proliferation of Vascular Smooth Muscle Cells Through Modulation of Various Steps of Growth Signals

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ABSTRACT: Among fatty acids, only n-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) inhibited the proliferation of vascular smooth muscle cells. DHA was less effective than EPA. To clarify the anti-proliferative effect of n-3 polyunsaturated fatty acid, we have explored the effect of EPA on the signal transduction pathway of platelet derived growth factor (PDGF). EPA inhibited PDGF binding on its receptor and activation of protein kinase C. EPA also suppressed c-fos mRNA expression, one of immediate early genes, through partly inhibiting c-fos transcription. These data revealed that EPA could inhibit vascular smooth muscle cell proliferation through modulating various steps of the signal transduction by PDGF.

Lipids 31, S-301–S-304 (1996).

Eicosapentaenoic acid (EPA) exerts its anti-thrombotic and atherogenic effect through the modulation of various cell functions related to atherosclerosis (1–3). We previously reported that only n-3 polyunsaturated fatty acids (PUFA) among fatty acids dose dependently inhibited the proliferation of rat cultured vascular smooth muscle cell (VSMC). Of these, EPA was most potent (4). We proposed that the change of membrane characteristics by EPA incorporation might modulate the signal transduction of growth factors. Here, we report how EPA modulates the signal transduction of growth factors, mainly platelet derived growth factor (PDGF). Growth signal of PDGF are transferred to nucleus through the activation of mitogen activated protein kinase (MAP-K) and protein kinase C (PKC). Special emphasis was placed on the effect of EPA on the signal transduction in the nucleus. Expression of immediate early growth gene, c-fos, and transcription of c-fos gene were studied.

MATERIALS AND METHODS

Materials. Fatty acid emulsions of EPA, docosahexaenoic acid (DHA), oleic acid, and linoleic acid were prepared as follows: 1,2,3 trieicosapentaenoil glycerol (98% pure, EPA–TG), synthesized by chemical condensation of glycerol and pure free EPA, was emulsified with phosphatidylcholine (PC), glycerol, tocopherol, and water according to the method of Geyer *et al.* (5). The emulsion contained 20.6% of EPA–TG, 2.3% of glycerol, 1.2% of yolk egg PC and 0.004% of tocopherol.

VSMC preparation. Rat cultured VSMC were prepared by ex-plant method as previously reported (6). VSMC were cultured by Dulbecco modified eagle medium (DMEM) with 10% fetal calf serum (FCS). Cells of passage fifth to tenth were used.

Proliferation of VSMC measured by ^{3}H-thymidine uptake. DNA synthesis was measured by incorporation of ^{3}H-thymidine (Thymidine, [Methyl-^{3}H]-(2.59–3.33 Tbq/mmol)) into VSMC. VSMC were cultured with growth medium for 60 h, and further incubated with FCS (-) DMEM containing various fatty acids-TG (40–160 μM) for 24 h and then stimulated with PDGF (PDGF-BB homodimer, human recombinant, 10–25 ng/mL; R&D Systems, Inc., Abingdon, Oxford, United Kingdom) for 24 h, and ^{3}H-thymidine uptake was measured for last 4 h. Radioactivity in TCA insoluble fraction was measured.

Fatty acid composition of total phospholipid of VSMC. VSMC were incubated with growth medium for 60 h, and further incubated with EPA–TG (40–160 μM) for 24 h (EPA-rich VSMC). Fatty acid composition of total phospholipid of the VSMC was measured by gas–liquid chromatography as previously reported (7).

PDGF binding on its receptor of VSMC. EPA-rich VSMC were incubated with various amounts of PDGF in the presence of 1 uCi of ^{125}I-PDGF (human recombinant PDGF-BB homodimer, >37 Tbq/mmol; Amersham International Co., Ltd., Amersham, Buckinghamshire, United Kingdom) for 3 h at 4°. Maximal binding site and Kd were obtained by Scatchard analysis.

Northern blot analysis for c-fos mRNA. EPA-rich VSMC were stimulated with PDGF (10–25 ng/mL) for 15–60 min. Then total RNA was isolated by AGPC (acid guanidium-thiocyanate-phenolchloroform), electrophoresed and transferred to nylon filter. Hybridization was carried out with ^{32}P-labeled probe (1.0-Kb rat c-fos cDNA fragment). Spots were quantified by Bio-image analyzer (Fuji Film Co., Ltd., Tokyo, Japan).

C-kinase activity. Particulate (membrane) and cytosolic (soluble) fractions were obtained after stimulating EPA-rich VSMC with PDGF (10 ng/mL) or 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (10^{-8}–10^{-7}M) for 15 sec to 30 min as follows: cells were scraped, sonicated, and centrifuged at

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Abbreviations: DHA, docosahexaenoic acid; DMEM, Dulbecco modified eagle medium; EPA, eicosapentaenoic acid; EPA–TG, trieicosapentaenoil glycerol; FCS, fetal calf serum; MAP-K, mitogen activated protein kinase; PC, phosphotidylcholine; PDGF, platelet derived growth factor; PKC, protein kinase C; PUFA, polyunsaturated fatty acid; TPA, 12-O-tetradecanoyl phorbol-13-acetate; VSMC, vascular smooth muscle cells.

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100,000 *g* for 60 min. PKC activity and protein amount of both fractions were determined with a PKC assay kit (Amersham) and western blotting using MC5 monoclonal antibody for PKC (Amersham), respectively.

Nuclear run-on assay. Nuclei from EPA rich VSMC treated with PDGF (10 ng/mL) or TPA (10^{-7} M) were prepared. Run-on transcription was carried out at 30°C for 30 min. Reaction was terminated and ^{32}P-labeled nuclear RNA was purified. The cDNA for c-fos, β-actin and plasmid vector (pSPT18) were denatured and applied to a nylon filter (Hibond-C) (10 μg per slot). Membranes were hybridized with ^{32}P-labeled nuclear RNA transcript for 48 h at 65°C. The blots were quantified by Bio-image analyzer. All experiments were performed at least twice and provided constant results.

RESULTS

EPA treatment dose dependently increased the content of EPA and docosapentaenoic acid without altering the arachidonic acid and DHA in total phospholipid of VSMC. EPA/arachidonic acid increased up to 6-fold (0.11; before treatment, 0.67; EPA 160 μM for 24 h) by EPA treatment as shown in Table 1.

DNA synthesis was enhanced up to 10–20 fold by the stimulation with optimal amount of PDGF (10–25 ng/mL). By the pretreatment of EPA–TG, DNA synthesis by PDGF was dose dependently suppressed. DHA–TG also showed a similar effect, but was less than that of EPA. Oleic acid and linoleic acid TG showed no effect on DNA synthesis as shown in Figure 1.

Nonspecific binding of ^{125}I- PDGF was less than 5% of total binding. Saturation analysis plots of the specific binding of ^{125}I-labeled PDGF revealed that EPA-TG pretreatment suppressed the binding of PDGF on its receptor of VSMC in a dose dependent manner. Scatchard analysis for PDGF receptor revealed a linear regression fit and one binding site. The Kd value was 286 fmol and maximum binding site (MXB) was 22.1 fmol/5 × 10^4 cells. EPA pretreatment suppressed the number of MXB in a dose-dependent manner (EPA 40 μM and 80 μM pretreatment; 17.4 and 14.6 fmol/5 × 10^4, respectively), but did not affect the Kd value. DHA showed a similar effect on MXB but oleic acid and linoleic acid showed no effect.

Activation of PKC was measured by evaluating the translocation of calcium-lipid dependent kinase activity or PKC protein amount from cytosol to particulate fraction in VSMC. Figure 2 shows the PKC activities recovered in the cytosol and particulate fractions of both control and EPA treated VSMC after stimulation with 10^{-7} M TPA. In basal conditions, PKC activity in both cytosol and particulate fractions did not differ between two groups of VSMC. Thirty seconds after stimulation with TPA, cytosol associated activity decreased rapidly and particulate associated activity increased without changing total PKC activity in both groups of VSMC. Particulate associated activity of EPA treated cells was less than that of control cells up to 30 min after stimulation by TPA. The similar difference was observed by measuring PKC protein amount in cytosol and particulate fractions after TPA stimulation in two groups of cells by western blotting. The translocation of PKC by PDGF was also suppressed in EPA treated cells.

TABLE 1
The Effect of Trieicosapentaenoic Glycerol Treatment on the Change of Fatty Acid Composition of Total Phospholipid of Vascular Smooth Muscle Cells[a]

	0 (μM)	40 (μM)	80 (μM)	160 (μM)
18:0	13.5 (1.0)	13.6 (1.2)	14.7 (2.0)	12.7 (0.3)
18:1 n-9	26.0 (8.6)	24.6 (7.5)	27.4 (7.3)	24.2 (7.1)
18:2 n-6	2.4 (1.2)	2.5 (0.9)	2.4 (1.1)	2.7 (1.0)
20:4 n-6	6.4 (1.7)	6.9 (1.3)	6.5 (2.2)	6.4 (1.0)
20:5 n-3	0.7 (0.2)	2.3 (0.4)	2.9 (0.7)*	4.1 (0.2)**
22:5 n-3	1.3 (0.1)	1.8 (0.1)	1.8 (0.4)	2.2 (0.3)
22:6 n-3	1.6 (0.3)	1.6 (0.3)	1.5 (0.5)	1.5 (0.4)

[a]The values shown are the mean (SE) of three cultures from one experiment. *$P < 0.05$, **$P < 0.01$, significantly different from zero dose values.

FIG. 1. Effect of various fatty acid triglycerols at the concentration of 0, 40, 80, and 160 μM on platelet derived growth factor (10 ng /mL) stimulated proliferation of vascular smooth muscle cells. n = 3–8, mean ± SE, **$P < 0.01$; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

The expression of c-fos mRNA maximally increased 30 min after PDGF or TPA stimulation. EPA treatment did not change the time course of c-fos mRNA expression. EPA treatment suppressed the expression of c-fos mRNA by PDGF in a dose dependent manner as shown in Figure 3. C-fos mRNA expression by TPA (10^{-7} M) was also significantly suppressed in EPA treated cells.

We next attempted to determine whether inhibitory effects of EPA on c-fos mRNA expression might result from changes in c-fos mRNA transcription or in the stability of c-fos mRNA. Nuclear run-on experiments were performed to establish whether this effect of EPA occurred at the transcriptional level. C-fos was not constitutively transcribed under serum starved conditions in VSMC. The transcription of β-actin, which served as a control of a constitutively expressed gene, was increased by serum or TPA stimulation. Almost no background hybridization of the transcripts with the vector

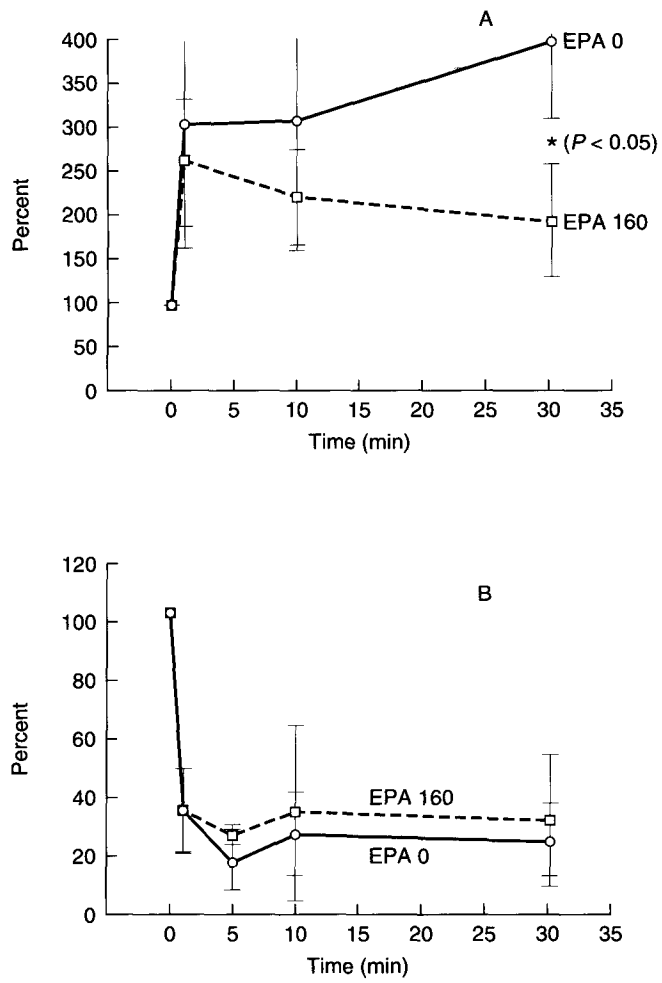


FIG. 2. Effect of 12-*O*-tetradecanoyl phorbol-13-acetate (10^{-7} μM) induced translocation of protein kinase C in vascular smooth muscle cell. n = 4, mean ± SD, *$P < 0.05$. See Figure 1 for abbreviation.

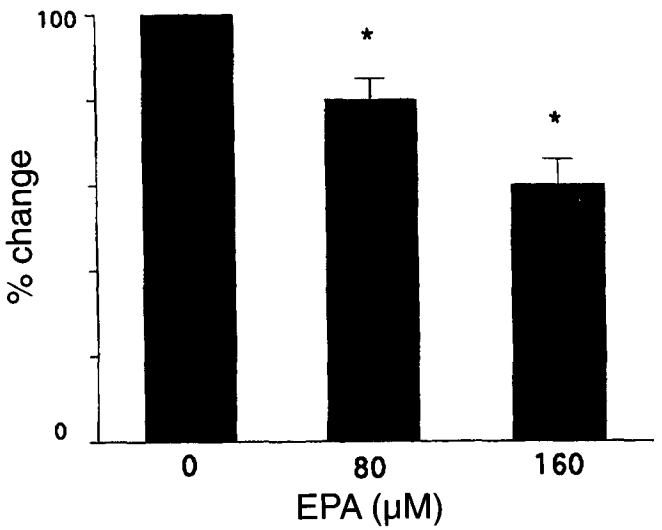


FIG. 3. Effect of eicosapentaenoic acid on platelet derived growth factor stimulated (10 ng/mL) expression of c-fos mRNA. n = 3, mean ± SE, *$P < 0.05$. See Figure 1 for abbreviation.

was detectable. 160 μM of EPA pretreatment inhibited the serum induced transcription of c-fos in VSMC.

DISCUSSION

Among PUFA, only n-3 PUFA inhibited the proliferation of VSMC. DHA was less effective than EPA and therefore we used EPA rich cells to clarify the mechanisms of PUFA to suppress VSMC proliferation (4). EPA and DHA suppressed the proliferation of VSMC possibly through the inhibition of binding of growth factors such as PDGF on its receptor. This might be derived from the change of membrane fluidity through the increase in PUFA such as EPA and DHA. PDGF binding on its receptor induces the activation of PDGF receptor through the autophosphorylation of receptor. This receptor phosphorylation leads to the activation of receptor linked several molecules, and this resulted in the activation of PKC and MAP-K. This finally induced the expression of early growth related genes including c-fos. C-fos mRNA expression is required for mitogen stimulated cell growth. EPA suppressed not only PDGF, but TPA stimulated c-fos mRNA expression in growth arrested cells in a dose dependent manner. This suggested that EPA could also act on the event after binding of growth factor on its surface receptor. In fact, EPA also directly suppressed the activation and/or translocation of PKC. PUFA are reported to regulate the expression of several genes involved in lipid metabolism and possibly regulate the PUFA response element within the proximal promotor region of some genes (8,9). EPA suppressed c-fos gene expression and transcription as demonstrated by northern blotting and nuclear run-on assay.

We first reported that the expression and transcription of growth related genes were inhibited by EPA. The effect of EPA on the promotor region of c-fos should be further clarified. EPA inhibited the proliferation of VSMC through the modulation of various steps of signal transduction by growth factor. These effects of EPA might partly explain the anti-atherosclerotic effects of EPA.

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The Structure-Activity Relationship of Lipoxygenase Products of Long-Chain Polyunsaturated Fatty Acids: Effects on Human Platelet Aggregation

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ABSTRACT: The effect of hydroperoxy and hydroxy derivatives of various fatty acids on human platelet aggregation was determined to delineate potencies and structure-activity function. In this regard, the 22-carbon n-3 fatty acids are the most potent inhibitors in comparison to the n-6 lipoxygenase derivatives. Submicromolar levels of the docosapentaenoic (22:5) and especially docosahexaenoic (22:6) n-3 hydroperoxy and hydroxy derivatives specifically antagonize the platelet aggregating effect to arachidonic acid (AA, 20:4n-6) but not that of ADP or collagen. Chain length (22-C > 20-C), double-bond position (n-3 > n-6), and double-bond number (6 > 5 > 4) influence the degree of inhibition of AA-induced aggregation of human platelets. Moreover, significant differences in potency were associated with specific structural aspects of 22:6n-3 lipoxygenase derivatives of 22:6n-3 as follows: functional group (OOH > OH) and positional isomer (14-OOH, 14-OH, 20-OOH > 11-OOH, 17-OOH > 10-OOH > 11-OH, 8-OOH, 7-OOH > 4-OOH).

Lipids 31, S-305–S-308 (1996).

It is well known that polyunsaturated fatty acids can be oxygenated by lipoxygenases to form hydroperoxy fatty acids, the first products of the lipoxygenase reaction, and subsequently hydroxy fatty acids. Mammalian platelets are high in lipoxygenase activity and capable of enzymatically producing a number of hydroxylated products of n-6 and n-3 fatty acids. For example, docosapentaenoic acid (22:5n-3) (1) and docosahexaenoic acid (22:6n-3) (2) are metabolized to 11- and 14-hydroxy positional isomers by 12-lipoxygenase. In addition, human platelets produce a 17-OH-22:6n-3 from 15-lipoxygenase, which is also present in the platelet (3). It has been proposed that the hydroxylated metabolites may be physiologically active in platelet-vascular smooth muscle and other cell interactions (4–6). A report by Sinzinger *et al.* (7) on the occurrence of myocardial infarct in young patients with platelet 12-lipoxygenase deficiency suggests involvement of hydroxylated derivatives in the regulation of platelet function. These metabolites may interfere with thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) receptors (8) and alter endogenous production of eicosanoids (9), thereby directly resulting in changes in cell function. Nutritional modification of the precursor fatty acid pool with n-3 or n-6 dietary supplementation or ethanol exposure alters the eicosanoid-docosanoid profile, which may result in altered cell function (10,11).

Hydroxylated fatty acids were enzymatically produced in platelets from 22:5n-6, 22:5n-3, 22:5n-5 and 22:6n-3, and hydroperoxidized 22:6n-3 fatty acids were prepared *in vitro* to perform a structure-activity study. Biological effects of hydroperoxidized and hydroxylated lipoxygenase products of n-3 and n-6 unsaturated fatty acids were characterized in human platelets.

MATERIALS AND METHODS

12-hydroperoxy-eicosatetraenoic (12-OOH-20:4n-6), 12-hydroxy-eicosatetraenoic (12-OH-20:4n-6), and 12-hydroxy-eicosapentaenoic acid (12-OH-20:5n-3) were purchased from Cayman Chemical (Ann Arbor, MI). 12-hydroperoxy-eicosapentaenoic acid (12-OOH-20:5n-3) was obtained from Calbiochem (La Jolla, CA).

Preparation of hydroperoxy 22:6n-3 derivatives. Autoxidation was performed using the procedure reported by Kim and Salem (12). Approximately 20 mg of 22:6n-3 (NuChek Prep, Elysian, MN) was dissolved in 1 mL of tetrahydrofuran in a 50-mL silanized glass, round-bottom flask. The solution was rotated under a stream of nitrogen to form a thin film of fatty acid. The flask was sealed and rotated overnight at room temperature after purging with 99.9% pure oxygen. Two more days of incubation at 5°C was required to yield a substantial conversion of hydroperoxy compounds. The contents were transferred to a silanized test tube using dichloromethane, dried under nitrogen, and redissolved in 1 mL cold methanol. An aliquot was diluted tenfold with methanol, and 20 µL was injected into a thermospray liquid chromatography/mass spectrometry (LC/MS) system for structural analysis. The total yield of hydroperoxy 22:6n-3 compounds, calculated using an extinction coefficient of 30,000 (13), was approximately 12.5%.

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Abbreviations: AA, arachidonic acid; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; TXA_2/PGH_2, thromboxane A_2/prostaglandin H_2.

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Preparation of soybean lipoxygenase 17-OOH- and 17-OH-22:6n-3 products. As previously reported (12), 2 mg of 22:6n-3 were dissolved in 20 μL ethanol. With vigorous vortexing, 4 mL 0.2 M borate buffer (pH 9) and then 500 μL enzyme solution (2×10^4 units/mL) were added. The mixture was incubated on ice for 30 min, then acidified with formic acid and extracted twice with dichloromethane. After being dried with a stream of nitrogen, the contents were redissolved in 1 mL methanol. An aliquot was diluted tenfold with methanol, and 20 μL was injected into a thermospray LC/MS system. The yield calculated from the ultraviolet absorbance of the products at 235 nm indicated that approximately 75% of the 22:6n-3 had been converted to the 17-OOH- and 17-OH-22:6n-3 derivatives.

Synthesis and analysis of hydroxylated 22-carbon fatty acids. Washed human platelets were prepared in a *tris*-EDTA buffer and incubated for 20 min at 37°C with 200 μM 22:5n-6, 22:5n-5, 22:5n-3, and 22:6n-3, as previously reported (14). Metabolites were then extracted twice with equal volumes of dichloromethane. Positional isomers were separated, purified and quantified using an HP-1090 high-performance liquid chromatograph (HPLC) equipped with a diode array ultraviolet detector (235 nm). A 4.6 × 25-cm Axxiom ODS column (5 μm; Thompson Instruments, Alexandria, VA) was used with a flow rate of 0.8 mL/min and a solvent system composed of 100 mM ammonium acetate and methanol. In some cases hydroxy fatty acids of interest were collected and methylated using diazomethane to analyze their structures (3).

Platelet aggregation. Human whole blood was obtained and diluted 5:1 in 3.8% citrate. Platelet aggregation to 20:4n-6 (0.25 mM) was evaluated using a Chronolog (Havertown, PA) whole blood impedance aggregometer. Whole blood samples (940 μL) were preincubated for 5 min with 50 μL of the vehicle (ethanol solution) or 50 μL of the various OOH- and OH-derivatives prior to the addition of 20:4n-6 (10 μL). The final concentration of ethanol in the platelet bath (<30 μM) did not alter platelet reactivity to 20:4n-6 at this concentration. Changes in aggregation were expressed as changes in the slope of aggregation (ohms/cm/min). In some cases, aggregating responses to ADP (2 μM; Sigma Chemical Co., St. Louis) and collagen (4 μg/mL; Sigma Chemical Co) were measured to test for specificity.

Statistics. All results were evaluated using analysis of variance and Student's *t*-test for unpaired samples. A P value of $P < 0.05$ was considered statistically significant.

RESULTS

Human platelets were preincubated with either indomethacin (0.5 mM) or 0.25 μM of the various OOH-22:6n-3, OH-22:6n-3, and OH-22:5n-3 isomers as well as other hydroxy fatty acids to determine their effect on arachidonic acid (AA)-induced (0.25 mM) platelet aggregation. The control-aggregating response to AA (20:4n-6, 0.25 mM) was generated following preincubation of the whole blood sample (950 μL) with 50 μL of the vehicle for 5 min. All other responses were referenced to this and are expressed as the change in slope. A typical control-aggregating curve is depicted in Figure 1A. Preincubation with indomethacin (0.5 mM) for 5 min blocked the aggregating response to 20:4n-6 via its blockade of the cyclooxygenase-generated mediator of aggregation, TXA_2 (Table 1). All hydroperoxy and hydroxy fatty acids tested reduced the slope of aggregation for AA. The most potent derivatives were the longer-chain (22-carbon) highly unsaturated (6 double bond) n-3 fatty acids, and the least potent were the shorter-chain (20-carbon) least-unsaturated n-3 and n-6 fatty acids (Table 1). Representative aggregating curves to 20:4n-6 are shown in Figure 1 in the absence and presence of the hydroperoxy fatty acids (A) and hydroxy fatty acids (B) tested.

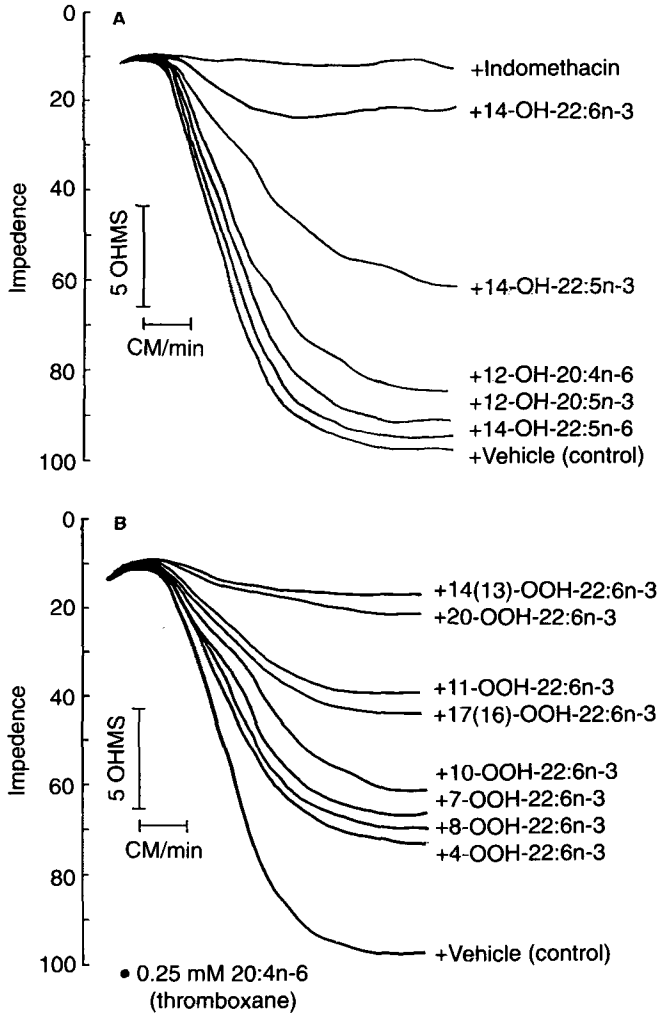


FIG. 1. A) Representative curves for arachidonic acid (20:4n-6)-induced (0.25 mM) human platelet aggregation in the presence of indomethacin (0.5 mM) and various hydroxy fatty acids (250 nM), in comparison to the control-aggregating response (vehicle-treated). B) Representative curves for arachidonic acid-induced (0.25 mM) human platelet aggregation in the presence of various hydroperoxy fatty acids (250 nM), in comparison to the control-aggregating response.

TABLE 1
Effect of Hydroperoxy and Hydroxy Fatty Acid (250 nM, 5 min) Pretreatment on the Slope of Aggregation for Arachidonic Acid (0.25 mM)

Pretreatment group	*N*	% Inhibition
Indomethacin (0.5 mM)	4	89 ±6[a]
14-OOH-22:6n-3	4	72 ± 8[a]
14-OH-22:6n-3	6	68 ± 6[a]
20-OOH-22:6n-3	4	67 ± 10[a]
11-OOH-22:6n-3	4	56 ± 11[b]
17-OOH-22:6n-3	4	54 ± 8[b]
10-OOH-22:6n-3	4	44 ± 10[b]
14-OH-22:5n-3	6	43 ± 7[b]
7-OOH-22:6n-3	4	40 ± 12[b]
8-OOH-22:6n-3	4	38 ± 12[b]
4-OOH-22:6n-3	4	37 ± 9[b]
12-OH-20:4n-6	6	21 ± 5[b]
12-OOH-20:5n-3	3	15 ± 10
12-OH-20:5n-3	6	14 ± 4[b]
14-OH-22:5n-6	4	11 ± 8
12-OOH-20:4n-6	4	10 ± 9

[a]Significance at $P < 0.01$.
[b]Significance at $P < 0.05$.

ADP- and collagen-induced platelet aggregation were not altered in the presence of the hydroperoxy or hydroxy 22:6n-3 fatty acids tested (12-OH-20:4n-6, 12-OH-20:5n-3, 14-OH-22:6n-3, and 14-OOH-22:6n-3).

DISCUSSION

Mammalian platelets contain an active lipoxygenase and are capable of enzymatically producing a number of n-6 and n-3 hydroxy fatty acids. Submicromolar levels of n-3 hydroperoxy and hydroxy fatty acids specifically antagonize platelet-aggregating effects of an AA metabolite, TXA_2. These are the first studies, to our knowledge, that identify a biological effect for these n-3 hydroperoxidized derivatives. With regard to structural features, the longer-chain 22-carbon metabolites of n-3 parent fatty acids with the greatest degree of unsaturation were the most potent inhibitors (i.e., 22:6n-3). These derivatives were at least one order of magnitude more potent in the platelet preparation compared to a recent report from our laboratory of similar inhibitory effects in vascular smooth muscle preparations (15). Albrand *et al.* (16) have demonstrated that the most stable conformation of the 22:6n-3 hydroxy derivatives are the coiled ones. Moreover, the most potent TXA_2 antagonist, the 14-hydroxy derivative of docosahexaenoic acid, exhibited stable conformations quite similar to those of TXA_2. Thus, specific conformations, in addition to the hydroperoxy and hydroxy functions and/or the presence of a conjugated diene, may be required for these derivatives to effectively interact with the TXA_2 receptor. Also, since the 14-OH derivative is more active than the 11-OH- and 17-OH 22 carbon n-3 positional isomers in both the present and previous studies (15,17), the position of this moiety appears crucial. Differences in potency were associated with specific structural aspects of the 22:6n-3 derivatives: the functional group (OOH > OH) and the position of oxygenation (14-OOH, 14-OH, 20-OOH > 11-OOH, 17-OOH > 10-OOH > 11-OH, 8-OOH, 7-OOH > 4-OOH) were modulating factors. These results argue for a relatively specific anti-TXA_2 activity of these lipoxygenase products.

Similarly, OH-22:6n-3 has been shown to block the TXA_2-induced decreases in cerebral blood flow and increases in mean arterial blood pressure measured *in vivo* in the rat (Karanian, *et al.*, unpublished data). Reported functional changes in platelet-aggregation in the present study may be attributed directly to antagonism at the platelet or vascular smooth muscle cell TXA_2/PGH_2 receptor. *In vitro*, OH-20:4n-6 has been shown to weakly inhibit $TXA_2/PGH2$-induced platelet-aggregation by interference at the receptor level (8). Moreover, 22-carbon n-3 parent fatty acids have been shown to inhibit both platelet-aggregation and TXA_2 binding (18,19). We have recently observed a dose-dependent inhibition of TXA_2 binding in platelets incubated with 14-OH-22:6n-3 with an IC_{50} of 0.9 μM (20). Analysis of binding parameters indicates that these derivatives induce a marked decrease in the affinity of the receptor for TXA_2 with a mild change in the number of receptor sites. Although less efficacious, inhibition of TXA_2 binding correlates strongly with inhibition of TXA_2-induced platelet-aggregation and is directly related to the concentration of hydroxy fatty acid (21). In the present study, we report that 0.25 μM 14-OH-22:6n-3 produced a 68% inhibition of AA-induced platelet-aggregation, whereas 0.9 μM produced a 50% inhibition of platelet TXA_2 binding. Changes in platelet TXA_2/PGH_2 receptor characteristics may only, in part, explain inhibitory effects of these derivatives.

Human platelets enriched with 22:6n-3 after a fish oil supplement produce small quantities of OH-22:6n-3 upon stimulation with thrombin (22). A more recent report from our laboratory, however, has demonstrated that the marked increase in the total lipid content of 22:6n-3 in rat platelets after an n-3–rich diet (23) correlated with a fourfold increase in OH-22:6n-3 production, to a level of approximately 340 picomoles (0.34 μM) per mL platelet suspension after stimulation with a calcium ionophore, A23187 (24). These recent data, taken together with the present findings, suggest that 22:6n-3 lipoxygenase derivatives may reach physiologically significant levels (>0.25 μM) with regard to platelet-vascular smooth muscle cell interactions involving TXA_2. Considering their relatively high potency as TXA_2 antagonists, they may contribute in part to the previously reported inhibitory effect of docosahexaenoic acid on AA or TXA_2-induced vascular contraction (25) and platelet-aggregation (18,26). Changes in cellular function, such as platelet-aggregation and smooth muscle contraction, may be regulated by the collective action of various lipoxygenase products of a number of polyunsaturated fatty acids.

22-Carbon hydroperoxidized lipoxygenase products may be physiologically active in platelet-vascular smooth muscle and other cell interactions. These metabolites may interfere with eicosanoid receptors (15) and alter the endogenous pro-

duction of eicosanoids (9), thereby directly resulting in changes in cell function. Although continued study of more potent hydroperoxidized analogues and their metabolism is necessary, these endogenously produced hydroperoxy/hydroxy derivatives may be involved in cardiovascular homeostasis and in certain pathophysiological states. Our understanding of the structure-activity relationships of these fatty acids may be of use in designing more efficacious TXA_2 antagonists. In this regard, these metabolites and related chemical forms may be of therapeutic value.

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Blood Pressure, Serum Lipids, and Fatty Acids in Populations on a Lake-Fish Diet or on a Vegetarian Diet in Tanzania

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ABSTRACT: Major risk factors for coronary heart disease were assessed in two populations of Tanzania, one on a fish diet (FD) living along the coast of Lake Nyasa, and the other, mainly on a vegetarian diet (VD), living in a farming area. Lower blood pressure values were found in the FD subjects (n = 618) vs. VD (n = 618) (systolic blood pressure, SBP, 120 ± 15 vs. 135 ± 20, $P < 0.01$; diastolic blood pressure, DBP, 70 ± 9 vs. 78 ± 11, $P < 0.01$, respectively). In an FD subgroup (n = 61), total cholesterol (TC) (122 vs. 136 mg/dL, $P < 0.01$); triglycerides (TG) (82 vs. 105 mg/dL, $P < 0.01$); and lipoprotein (a) [Lp(a)] (19.9 ± 18.4 vs. 32.3 ± 22.4, $P < 0.001$) were lower than in a VD subgroup (n = 55). Serum fatty acids (FA) in the FD subgroup were as follows: eicosapentaenoic acid (EPA) (20:5) 2.48 vs. 0.72%, docosahexaenoic acid (DHA) (22:6) 5.93 vs. 1.49%, vs. the VD, respectively. Arachidonic acid (AA) (20:4n-6) also was higher in the FD vs. the VD group (9.85 vs. 8.30%, $P < 0.05$), whereas 18:2n-6 was about double (23.97 and 14.85%) in VD vs. FD. The peculiar serum FA pattern in FD reflected the FA of dietary fish. In fact, in four main species of lake fish, DHA was 8–19%, higher than EPA (1.8–4.2%), in contrast with the situation in cold-water fish, and AA was 5.8–8%, higher than in cold-water fish. The data, obtained in populations strictly on natural, unprocessed, low-fat diets, show that a diet based on freshwater fish results in lower BP, serum TC, TG, and Lp(a), and suggests that serum AA is not reduced when the major dietary n-3 is DHA rather than EPA.

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Comparative studies in populations living on different diets have provided information on correlations between dietary fat and incidence of coronary heart disease (CHD). Consumption of manufactured foods in economically advanced countries, however, minimizes dietary differences, and comparisons among populations are limited by confounding (e.g. genetic) factors. Studies in genetically homogeneous populations that are living on differing diets are therefore quite useful. The World Health Organization (WHO) is involved in studies aimed at assessing the comparative incidence of CHD risk factors in populations living in areas with distinctly different dietary patterns. A study was thus set up by WHO and coordinated by the Institute of Clinical Medicine, University of Padua, (Italy), aimed at evaluating blood pressure (BP) and serum lipids in populations on different diets, in the Lugarawa district of Tanzania. The study was centered on two areas, one (Lupingu) on the shores of Lake Nyasa, and the other (Madilu), a distance of about 90 mi from the lake. Processed foods are not available, and the diet strictly reflects local food sources. The population living in the coastal area eats mainly fish (fish diet, FD); whereas, in the rural area, grains, potatoes, and vegetables are the main components of the diet (vegetarian diet, VD). Relationships between serum fatty acid (FA), reflecting the composition of dietary fat, and BP and serum total cholesterol (TC), triglyceride (TG), and lipoprotein(a) [Lp(a)] levels were investigated. In addition, correlations between metabolically related FA in serum lipids in the two populations were evaluated and the FA compositions of the four major edible species of lake fish were analyzed. This paper is mainly focused on serum FA profiles and on relationships among individual FA of the n-6 and n-3 series in the two population groups. The major observations are that levels of BP, TC, TG, and Lp(a) were significantly lower in the fish-eating population. n-3 FA, especially 22:6 (docosahexaenoic acid, DHA), was higher in plasma lipids of subjects on the fish diet, as expected, but in this group, levels of 20:4n-6 (arachidonic acid, AA) were also higher than in people living inland.

MATERIALS AND METHODS

BP and Lp(a) measurements were made on large population groups (over 600 subjects) in each area, whereas determinations of serum TC, TG, and serum FA profiles were carried out on a smaller, representative population, due to difficulties in analysis of a larger number of samples. The study was

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Abbreviations: AA, arachidonic acid; BP, blood pressure; CHD, coronary heart disease; DBP, diastolic blood pressure; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; FD, fish diet; Lp(a), lipoprotein(a); PUFA, polyunsaturated fatty acids; SBC, systolic blood pressure; TC, total cholesterol; TG, triglycerides; VD, vegetarian diet.

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based on collaboration with the rural hospital in Lugarawa, Tanzania, which participates in the medical programs of the International College for Health Cooperation (CUAMM).

Subjects. A total of 654 (368 men and 286 women) in Madilu (on a VD) and of 622 (370 men and 252 women) subjects in Lupingu (on a FD) were recruited.

Dietary assessment. Dietary survey was carried out through questionnaires collected from 25 families in each village. Dietary constituents were calculated using standard food tables (1). For collecting data, since the questionnaire adopted in the World Health Organization (WHO) MONICA study (2) could not provide reliable data on individual diets, except for salt supplementation, an accurate assessment of food consumption and composition was obtained in each family. In this manner, an indirect estimate of individual diet was obtained. Calculated daily average intakes for the VD were as follows: total calories, 1,357–2,860 kcal: 41% from maize (~250 g), 17% from rice (~100 g), 15% from beans (~100 g), 6% from potatoes (~100 g) and bananas (~140 g), and ~13% from low-alcohol beverages (1 L). Calculated daily average intakes for the FD were as follows: 1,553–2,838 total calories: 23% from fish (~500 g), 38% from tapioca (a starch obtained from the root of the cassava) (~250 g), ~7% from beans (~50 g), 6% from rice (~40 g), and 9% from low-alcohol beverages (~0.75 L). Salt intakes were 4.4 ± 1.5 g/d in FD vs. 4.0 ± 1.4 in VD, respectively.

Statistical analysis. Comparisons between groups were made by Student's *t*-test analysis. Correlations were evaluated using Pearson's coefficient.

Measurements. All subjects were subjected to BP measurements and blood drawing. Frozen serum samples were shipped to Italy; Lp(a) was measured in all samples, whereas 61 (26 men and 35 women) FD and 55 (24 men and 31 women) VD randomized samples were analyzed for lipid levels and FA profiles. FA analysis also was carried out on samples of typical lake fish. TC and TG were measured by standard techniques, and Lp(a) concentrations were determined by immunoassay (3). FA of serum lipids and of fish from Lake Nyasa were analyzed by capillary gas chromatography. Serum FA of 16 Italian subjects, 9 males and 6 females, aged 25–42, as reference samples, representative of a population on a Western diet, were also analyzed.

RESULTS

Serum TC, TG, Lp(a), and BP were significantly lower in the FD than in the VD (Table 1). Major differences, some unexpected, between the two groups were observed in major serum n-6 and n-3 polyunsaturated fatty acids (PUFA), reported in Table 2, together with levels in Italian subjects taken as reference values. Levels of 18:2n-6 were higher and those of n-3 FA lower in VD than in FD. In FD, despite elevation of n-3 FA, AA was, surprisingly, even higher than in VD, and, among the n-3, DHA was considerably elevated over the level of EPA. In the Italians, levels of 18:2 n-6 and of 20:4 n-6, were the highest and the lowest respectively, of the three groups, whereas n-3 FA levels were comparable to those in the VD group. The ratio of n-3 to n-6 FA in the FD group was about four- and eightfold that in VD and in Italians, respectively. The combined elevation of both AA and, among n-3, especially DHA, in the FD group, prompted us to analyze the FA in samples from various fish species from the lake, typically eaten by the FD group. These values (Table 3) were compared with those reported for a tropical fish and for North Atlantic mackerel. The fish species have a different total lipid content, but all contain appreciable AA and DHA concentrations higher than those of EPA. These profiles resemble those in the Equatorial Indian halibut and other tropical species (4), and are different from those of cold-water fish (5). Calculated intakes of AA in the FD group are in the range of 0.4–0.8 g/d. Although we do not yet have detailed data on fat intake, it appears that serum FA in the FD group reflected the composition of fish FA, i.e., levels of both AA and DHA were relatively high. Correlation analysis reveals that, in the n-6 sequence, serum levels of 20:3 and 18:2 (Δ6 desaturation + elongation) were correlated in both groups (Table 4), but AA was correlated with 18:2 and 20:3 (Δ5 desaturation) only in VD. In the n-3 sequence, there was a significant correlation between EPA and 18:3 in VD, but not between DHA and 18:3, or between DHA and EPA. In FD, however, there was only a correlation between DHA and EPA.

DISCUSSION

This study shows that, in genetically homogeneous population groups living on distinctly different diets, the incidence

TABLE 1
Levels of Serum Total Cholesterol, Triglycerides, Lipoprotein(a), and Systolic and Diastolic Blood Pressures in Two Populations[a]

	TC (mg/dl)	TG (mg/dL)	Lp(a) (mg/dL)	SBP (mmHg)	DBP (mmHg)
FD	121.6 ± 30.9	80.6 ± 40.7	19.9 ± 17.7	120.4 ± 15.1	70.5 ± 8.9
	(*n* = 61)	(*n* = 61)	(*n* = 618)	(*n* = 618)	(*n* = 618)
VD	136.3 ± 39.8	105.3 ± 53.1	32.3 ± 22.4	134.7 ± 20.4	77.6 ± 10.6
	(*n* = 55)	(*n* = 55)	(*n* = 645)	(*n* = 645)	
P	<0.05	<0.001	<0.001	<0.001	<0.001

[a]TC = Total cholesterol; TG = triglycerides; Lp(a) = lipoprotein (a); SBP = systolic blood pressure; DBP = diastolic blood pressure; FD = fish diet; VD = vegetarian diet.

TABLE 2
Major Polyunsaturated Fatty Acids in Two Populations and in Italian Subjects[a]

	Bantu population		Healthy Italian subjects
	VD	FD	
Major PUFA[b]	(n = 55)	(n = 61)	(n = 16)
18:2n-6	23.97 ± 4.37	14.85 ± 4.30	34.55 ± 7.96[c]
20:4n-6	8.26 ± 1.94	9.85 ± 2.68	7.00 ± 1.20[c]
20:5n-3	0.72 ± 0.22	2.48 ± 1.35	0.64 ± 0.32
22:6n-3	1.49 ± 1.04	5.93 ± 1.77	1.39 ± 0.36
Total n-6	35.37 ± 5.73	27.31 ± 4.91	43.70 ± 5.12
Total n-3	3.50 ± 1.23	10.11 ± 3.25	2.34 ± 0.75
n-3/n-6	0.10 ± 0.04	0.38 ± 0.13	0.05 ± 0.02

[a]Values (weight percentages) are average ± SD. All values in FD are significantly different from VD and Italians ($P < 0.001$). Abbreviations as in Table 1.
[b]PUFA = Polyunsaturated fatty acid.
[c]Significantly different from VD and FD ($P < 0.01$).

TABLE 3
Lipid Contents and Percentage Levels of Major PUFA of Four Fish Species from Lake Nyas[a]

	Kambale[a]	Mfui[a]	Njenu[a] (Carp)	Mbelele[a] (Catfish)	Equatorial Indian Halibut[b]	N. Atlantic Mackerel[c]
Fat (g%)	1.8	1.1	4.9	10.3	1.7	(Oil)
Fatty acid						
20:4n-6	5.9	8.0	5.8	4.3	6.3	0.4
20:5n-3	2.1	3.1	1.8	4.2	5.9	7.6
22:5n-3	5.2	3.7	5.0	1.8		
22:6n-3	13.3	19.1	7.8	8.6	10.4	7.7

[a]Local names.
[b]See Reference 4.
[c]See Reference 5.

TABLE 4
Product-Precursor Correlations Between Major n-6 and n-3 Polyunsaturated Fatty Acids in Serum of Two Populations[a]

	VD		FD	
	r	*P*	*r*	*P*
n-6				
18:3 vs. 18:2	0.027	n.s.	0.210	n.s.
20:3 vs. 18:2	0.369	<0.01	0.528	<0.001
20:4 vs. 18:2	0.389	<0.01	0.155	n.s.
20:4 vs. 20:3	0.437	<0.001	0.063	n.s.
n-3				
20:5 vs. 18:3	0.301	<0.01	0.217	n.s.
22:6 vs. 18:3	0.117	n.s.	0.117	n.s.
22:6 vs. 20:5	0.166	n.s.	0.633	<0.001

[a]Abbreviations are as in Table 1; r = correlation coefficient; n.s. = not significant.

of major CHD risk factors is different. Various components in the diets, which need to be defined and quantified, may be responsible for this; one major difference concerns consumption of fish, and this is reflected in the serum FA profiles. FA profiles of the four lake-fish species resemble those of tropical fish (3,4) and are quite different from those of nontropical fish (5). High levels of both AA and of n-3 FA, especially DHA, in serum of FD, therefore, clearly reflected the composition of fish FA. Relatively low levels of EPA in fish may partly explain why AA levels in serum of the FD group were not reduced, since EPA competes with AA more effectively than DHA for esterification into lipids. Some as yet unspecified characteristics of the diet, such as a rather low fat intake, may also be important in affecting serum PUFA profiles. For instance, levels of both AA and n-3 FA in the VD group, on a diet providing low intakes of preformed long-chain PUFA, were comparable to those in the Italian subjects, on a typically omnivorous diet. This suggests efficient biosynthesis from the 18-carbon precursors in VD, as supported also by the good product-precursor correlations in serum FA. An additional observation is that, despite high levels of AA in serum, n-3 FA still exerted beneficial effects, e.g., on BP, suggesting that these were not mediated by interference with AA utilization and with the eicosanoid system. The lower serum TG levels, and to some extent, the lower BP values in the FD group, were predictable, although differences in BP between FD and VD subjects were greater than expected from data in clinical studies (7). Additional and undisclosed dietary factors may contribute to amplify effects of the fish diet, but differences in salt intake were excluded. Finally, levels of Lp(a), an independent risk factor for CHD (6), were significantly lower in FD. Lp(a) levels are determined mainly genetically, although dietary factors appear to elevate, [e.g., *trans* (9) and saturated FA (8)], or reduce, [n-3 FA (11,12)], this variable. Unsuspected genetic factors may, however, explain different Lp(a) values in the two groups. In conclusion, the Lugarawa study shows marked differences in the incidence of major CHD risk factors in two population groups living on distinctly differing simple and unprocessed foods. In addition, consumption of fish with elevated levels of both AA and n-3 FA still results in beneficial influences on various physiological variables.

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n-3 Fatty Acids Decrease Colonic Epithelial Cell Proliferation in High-Risk Bowel Mucosa

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ABSTRACT: The n-3 fatty acids (C20:5, eicosapentaenoic acid; C22:6, docosahexaenoic acid) may be important in the development, growth, and metastasis of colon cancer, a leading cause of death in North America. Patients who have had a bowel neoplasm have a high risk of developing a second neoplasm, and this risk is associated with a high percentage of cells correspond to the S phase of bromodeoxyuridine (BrdUrd) labeling in mucosal epithelial cells. To determine the effect of n-3 fatty acid supplementation on DNA synthesis of rectal mucosa, patients with stage 1 or stage 2 colon carcinoma or adenomatous polyps were randomized to consume either 9 g/d n-3 fatty acid capsules or 9 g/d placebo capsules. Plasma phospholipid fatty acid analysis and proctoscopic mucosal biopsies were performed at baseline, 3, and 6 mon. Colonic crypts were isolated from the mucosa, disassociated with enzymes, and incubated with BrdUrd, and %S phase was measured by flow cytometry. The plasma phospholipid n-6/n-3 ratio was determined by gas chromatography. Supplement compliance was assessed by plasma phospholipid n-6/n-3 ratio. Mean capsule consumption in these two group was 82%. Prior to supplementation, there were no significant differences in the %S phase and the plasma n-6/n-3 ratio between these groups. Patients whose colonic epithelial cells indicated hyperproliferation at baseline showed a strongly positive correlation to the %S phase of BrdUrd uptake and the n-6/n-3 ratio. There was no significant change after n-3 treatment in patients with low baseline. Those in the placebo group showed no significant difference in n-6/n-3 ratio, although there was an increase in the %S phase of BrdUrd uptake at 6 mon. The n-3 group did not have significant side effects, and polyps were not found after completing 12 mon of n-3 fatty acid supplementation. This study suggests that n-3 fatty acid may be a useful chemopreventive agent in some patients as reflected in a plasma biomarker of colon tumor growth and metastasis. A low plasma phospholipid n-6/n-3 fatty acid ratio may serve as a nutritional marker that is associated with colonic epithelial cell hyperproliferation in the n-3–supplemented group as compared with the placebo group.Characteristics of mucosal proliferation at baseline may be a crucial factor for the effect of n-3 fatty acid supplementation.
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Abbreviations: BrdUrd, bromodeoxyuridine; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FITC, fluorescein isothiocyanate; LI, labeling index.

An estimated 133,500 cases of colorectal cancer were diagnosed in the United States in 1996 (1). Colorectal cancer is the second leading cause of cancer mortality and will result in over 54,900 deaths in the United States. Epidemiological and laboratory animal studies appeared that linked high fat consumption with colorectal cancer (2). The low colon cancer rate among Alaskan and Greenland Eskimos has been associated with high consumption of fish (3,4). Animal studies indicated that the primary effect of fat is exerted on the promotion stage of carcinogenesis, with the n-6 fatty acid having been recognized as a major promoter (5–7), whereas fish oil containing the n-3 fatty acids eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3) are found to reduce tumor incidence, growth, and metastasis (8–11). The proposed pathophysiological mechanisms of fish oil protective effects include influences on the mucosal prostaglandin synthesis, reducing ornithine decarboxylase and tyrosine protein kinase activities, which has been shown to exert a proliferative effect on the colonic mucosa (12,13).

Colorectal adenomatous polyps with abnormal cell–kinetics characteristics has been suggested to be putative precursor lesions to carcinoma (14). Bruce *et al.* (15) and Lippmann *et al.* (16) showed that hyperproliferation of colonic epithelium is an appropriate intermediate end-point for chemopreventive intervention.

By the support of prior evidence, we conducted this clinical trial by use of objective flow cytometric assay. The aim of our study was to investigate the tolerance and effect of long-term n-3 fatty acid supplementation on DNA synthesis in high risk bowel mucosa and the efficacy of the plasma phospholipid n-6/n-3 ratio as a nutritional marker on the prevention of colonic tumor development and metastasis.

EXPERIMENTAL PROCEDURES

Patients with resected Dukes's A or B adenocarcinoma of the colon or rectum or severely dysplastic adenomatous polyps were recruited. All patients were eligible only after complete excision of their cancers as well as of any associated polyps. Twenty-seven patients were (2:1) randomly assigned to double-blind treatment groups: 17 for the n-3 fatty acid group and 10 for the placebo group. The n-3 capsules were composed of IND #041821 (NAOO, Charleston, SC), an n-3 ethyl ester

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concentrate prepared from vacuum-deodorized menhaden oil using transesterification, urea adduction, and short-path distillation. The concentrate contained approximately 80% n-3 fatty acid ethyl esters (44% EPA, 24% DHA, 10–12% other n-3 fatty acid ethyl esters). The placebo ethyl ester (NAOO) was prepared from corn oil. The preparation contained approximately 50% linoleic ethyl ester, 10% C_{16}, 15% other C_{18} fatty acid ethyl esters. Both capsules contained 0.2 mg/g 1,5-di-*tert*-butyl-1,4-benzohydroquinone as antioxidant, 2 mg/g tocopherols, and 2.0 mg/g cholesterol. Cholesterol was added to the placebo capsules to match the n-3 capsules. Patients were required to consume 9 capsules per day, 1 g per capsule.

Cell proliferation study: BrdUrd incorporation. Mucosal tissue obtained by proctoscopic mucosal biopsy was incubated for 20 min in 1.5 mM EDTA and 1.3 mM dithiothreitol in calcium-free, phosphate-buffered saline to separate crypt cells. After the suspension was precipitated on ice, crypt cells were prepared for enzymatic digestion. The cell suspension was filtered through a 0.22 μm sterile membrane. Single-cell suspensions were incubated at 37°C in RPMI (pH 7.2–7.4) containing 5% fetal calf serum, 20 μM fluorodeoxyuridine, and 30 μM BrdUrd for 1 h. After BrdUrd labeling, the cells were fixed in ice-cold 70% ethanol. Ethanol-fixed cells were added to 2 N HCl with 0.2% pepsin to denature the DNA for detection of BrdUrd by the fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody. After incubation for 40 min in the dark, the cells were centrifuged and neutralized with 0.1 M sodium tetraborate (pH 8.5). Cells were washed and incubated with 20 μl of FITC-conjugated anti-BrdUrd antibody (Becton Dickinson, San Jose, CA) for 45 min in the dark. Nuclei labeled with BrdUrd-anti-BrdUrd-FITC were then washed and resuspended in 0.01 mg propidium iodide. After 15 min of staining, cells were ready for analysis by flow cytometry.

Flow cytometry. Cytometric analysis was performed on a fluorescence activated cell sorter (FACS) (Becton Dickinson, Mountainview, CA). Cells were excited with a 15 mW laser at 488 nm. Red fluorescence was collected through a 585-nm bandpass filter and recorded as the total amount of DNA. Green fluorescence of the BrdUrd-labeled cells was collected through a 530-nm bandpass filter and recorded as the amount of incorporated BrdUrd. Data were collected in a 64 x 64 channel distribution showing the total DNA (red) against the logarithmic amount of incorporated BrdUrd (green).

Fatty-acid analysis. Plasma phospholipid fatty acid was extracted by use of a 2:1 mixture of chloroform/methanol. Hepatadecanoic acid (Nu-Chek-Prep, Elysian, MN) was added as an internal standard prior to extraction. The chloroform layer was dried, and thin-layer chromatography with LK5D silica gel plates (Whatman Chemical Separations, Inc., Clifton, NJ) was applied to separate various lipid fractions. The solvent system used to develop the plates was a mixture of petroleum ether/diethylether/glacial acetic acid (80:20:1). Phospholipid fractions were scraped and placed into teflon-lined, screw-cap glass tubes and hydrolyzed and methylated under nitrogen in a steam bath for 45 min by 14% boron/trifluoride in methanol (Alltech Associates, Deerfield, IL).

Gas–liquid chromatography. Fatty acid methyl esters were separated and quantified by use of a Hewlett Packard (Palo Alto, CA) 5890 Series II gas chromatograph equipped with a 30-M fused silica capillary column (0.32 mm I.D., 0.20 μm film thickness, Supelco, Bellefonte, PA) and a flame ionization detector. Helium was used as the carrier gas with a flow rate of 5 mL/min. The split ratio used was 14.3:1 with injector and detector temperatures at 240°C. Oven temperature was programmed to start at 70°C, then rise to 130°C at a rate of 10°C/min; then to 150°C at a rate of 2°C/min; then reach 195°C at a rate of 3°C/min. Peaks were identified by comparison to authentic standards (Nu-Chek-Prep) by a Hewlett Packard 7673 controller. Quantitation was based on the relative responses of the internal standard and fatty acid methyl esters in the sample as compared with the responses of standards. Results were expressed in absolute concentrations (μmol/L plasma) as well as a relative percent of the total fats analyzed.

RESULTS

Fatty acid analysis of the plasma phospholipid showed that EPA and DHA contents increased over 6 mon in the n-3–supplemented group, whereas levels of linoleic acid and arachidonic acid decreased (Figs. 1 and 2). The mean of the plasma n-6/n-3 ratio in the n-3 group decreased over 6 mo. Unlike the n-3 group, the placebo group showed no significant changes in the concentration and relative percent of EPA, DHA, linoleic acid, arachidonic acid, and the n-6/n-3 ratio (Figs. 3 and 4).

The BrdUrd labeling index (LI) of colonic mucosa in the n-3 group showed a strongly positive correlation to the plasma phospholipid n-6/n-3 ratio. It is interesting that there was a wide range of BrdUrd labeling index among subjects before starting this trial. From the pattern of mucosal proliferation at baseline, it can be classified into two phenotype categories: high percent of BrdUrd-labeled cells in the S phase relative to the total number of cells examined (>10%S phase of BrdUrd LI, n = 7) and low proliferation (<10%S phase of

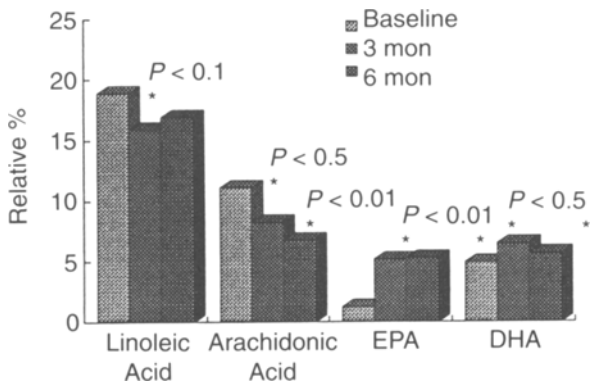


FIG. 1. Plasma phospholipid fatty acid profile of patients with high baseline proliferation on n-3 fatty acid supplement; *P* values were analyzed by Student's *t*-test; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

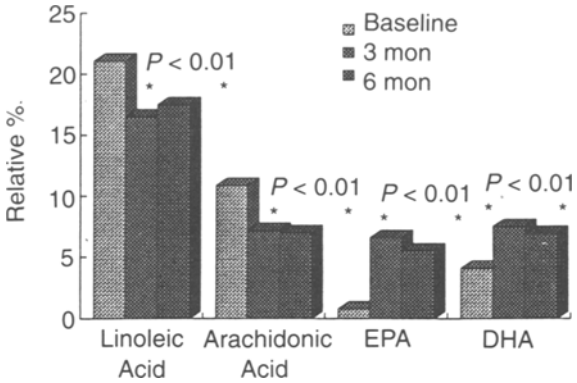


FIG. 2. Plasma phospholipid fatty acid profile of patients with low baseline proliferation on n-3 fatty acid supplement; *P* values were analyzed by Student's *t*-test. Abbreviations as in Figure 1.

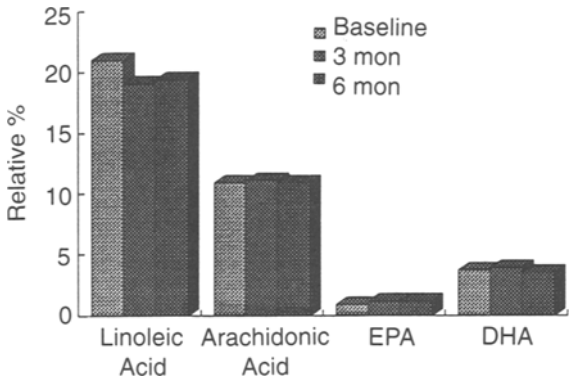


FIG. 3. Plasma phospholipid fatty acid profile of patients on placebo. Abbreviations as in Figure 1.

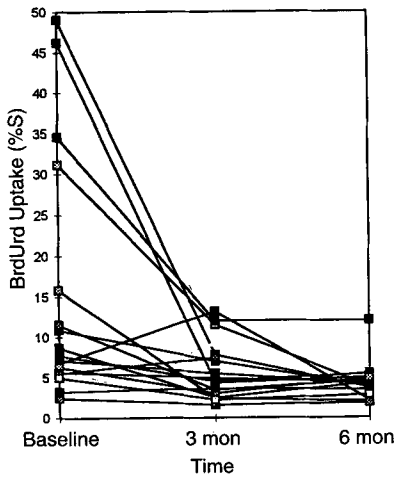


FIG. 4. Plasma n-6/n-3 and proliferation rate on placebo group.

BrdUrd LI, n = 10). It is important to note that the responses of decreasing BrdUrd LI to n-3 fatty acid supplementation mostly resulted from reductions of patients whose baseline patterns showed abnormal or hyperproliferation (Fig. 5). All

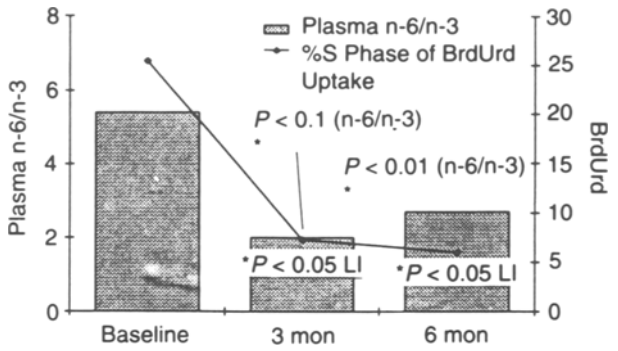


FIG. 5. Bromodeoxyuridine (BrdUrd) uptake in n-3 group.

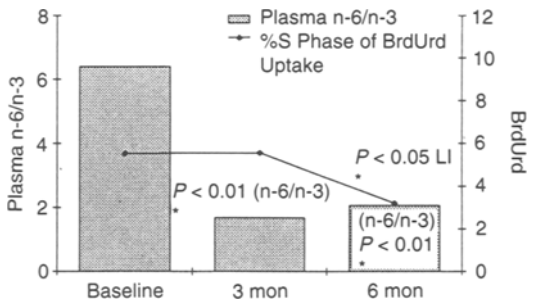


FIG. 6. Patients with high baseline proliferation on n-3 fatty acid supplement; *P* values were analyzed by Student's *t*-test. Abbreviation as in Figure 5.

hyperproliferative patients were found to have significantly decreased BrdUrd LI after 3 mon of n-3 treatment. In contrast, there was no dramatic change over time in those with low baseline LI in n-3. Subjects with high baseline LI decreased the mean proliferation rate of epitheial cells from 25.5 to 7.3 at 3 mo, along with the decline of the plasma n-6/n-3 ratio from 5.4 to 2.0. (Fig. 6). In contrast, other subjects with low baseline tended to maintain the proliferation rate, even the plasma n-6/n-3 ratio significantly decreased from 6.4 to 1.7 at 3 mon (Fig. 7). In the placebo group, the %S phase of BrdUrd uptake was increased from less than 6 to 9% at 3 mon, and to over 10% at 6 mon (Fig. 7). Despite the additional 4.5 g per d of linoleic acid consumption, the plasma n-6/n-3 ratio in the placebo group showed no significant difference. In addition, prior to supplementation, there were no significant differences in the plasma phospholipid n-6/n-3 ratio between these groups.

The fish oil supplement was well tolerated by study participants. There were no side effects, and no additional polyps were found in 12/12 patients who completed 12 mon of n-3 supplementation. Plasma fatty acid levels and capsule counts (82%) indicated good overall compliance. None of the patients experienced significant body weight gain or loss or any other physiological changes. Responses of the fatty acid profile and mucosal proliferation rate to n-3 was noticed as soon as three months after treatment started.

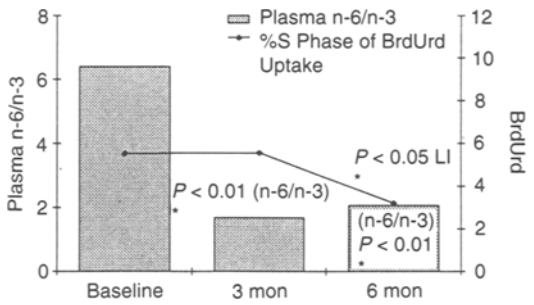


FIG. 7. Patients with low baseline proliferation on n-3 fatty acid supplement; *P* values were analyzed by Student's *t*-test. Abbreviation as in Figure 5.

DISCUSSION

In our study, the plasma phospholipid n-6/n-3 ratio successfully predicted responses of mucosal LI to fish oil supplementation. Increased incorporation of 22:6n-3 (DHA) and 20:5n-3 (EPA) and decreased incorporation of 20:4n-6 (AA) are reliable indices of n-3 supplement ingestion. Previous studies also showed that high fish oil intake altered the plasma fatty acid profile and incorporation of n-3 fatty acids into colonic mucosal cells (17). The plasma fatty acid profile has been shown to parallel the tissue status (18). Due to limitation of obtaining rectal biopsy specimens, we developed this plasma phospholipid n-6/n-3 ratio as a nutritional compliance marker. This new finding can be beneficial for the future design of fish oil chemoprevention trials.

Patients in the n-3 group experienced significant reductions in the BrdUrd LI. The effect of n-3 fatty acids was found exert only in patients with hyperproliferative patterns in this study. It seems that patients who had abnormal patterns of colonic cell proliferation benefited more from fish oil supplementation. Occurrence of adenomatous polyps in those patients who have normal baseline patterns may be not related to different types of fat intake. It has been suggested that more than one genetic pathway leads to the development of adenocarcinoma of the colon and rectum (19). It is possible that the effect of fatty acids on lowering abnormal proliferation is only capable in hyperproliferation. Anti *et al.* (18) previously reported a similar discrepancy among subjects' proliferation patterns at baseline. Our data shows that the LI of hyperproliferation could reach more than 30% BrdUrd LI, although previous data indicated that the highest percentage of LI was only in the low 20s by use of [^{3}H]thymidine autoradiography. Unlike Italian diets based on olive oil, which is low in linoleic acid, however, North American diets consume much more n-6 polyunsaturated fatty acid, which consists of more than 50% safflower, sunflower, soybean, corn, and cottonseed oils (19, 20). In our study, there was approximately 15 g/d of linoleic acid (18:2n-6) in the average diet of 30% fat content in both groups. Because of the competition between n-3 and n-6 for eicosanoid formation in the phospholipid of cell membranes, it is possible that chemoprevention studies conducted in North America need a longer period of treatment and a higher supplemental dose of n-3 fatty acids (21).

In conclusion, these cases suggest that n-3 fatty acids may be useful chemopreventive agents, as reflected in a plasma biomarker of colon proliferation tumor growth and metastasis. A decrease in the plasma phospholipid n-6/n-3 ratio can serve as a nutritional marker associated with colonic epithelial cell proliferation in the n-3–supplemented group compared with the placebo group. More studies must be undertaken, however, to test whether n-3 fatty acids are effective in inhibiting mucosa neoplastic proliferation in various genetic pathways.

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